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THE UNIVERSITY OF ALBERTA

Studies on Cyclosporine Analogs, Asymmetric Synthesis of α-Hydrazinocarboxylic Acids and Biosynthetic Studies Using Stable Isotope NMR Techniques

by

Laird Alexander Trimble

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Department of Chemistry

EDMONTON, ALBERTA

Fall 1986

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Supervisor

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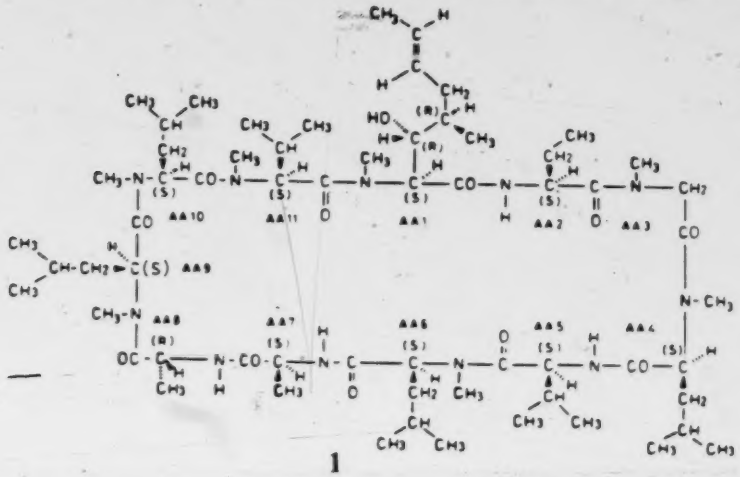
External Examiner

Date September 25, 1986.

To my father

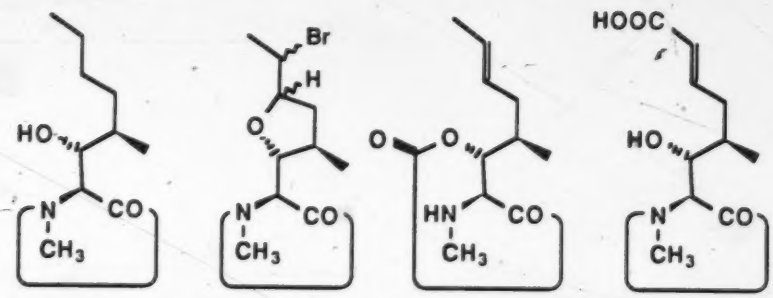
Abstract

Cyclosporine (**1**), a fungal metabolite isolated from *Tolypocladium inflatum* contains the unusual amino acid MeBmt (AA-1). Compound **1** exhibits a strong



1

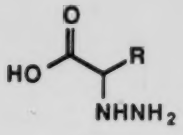
immunosuppressive effect in humans because it inhibits lymphokine interleukin II (IL-2) production at the level of translation. It is the drug of choice in organ transplants, particularly those of the kidney and liver. Since little is known about the binding of cyclosporine to its receptor, several analogs such as **2**, **7** and **14** were prepared.



2 **7** **14** **17**

The biological effect of these analogs was estimated by Dr. Chris Bleackley (University of Alberta) by measuring the reduction of IL-2 production in mouse and human cell lines. It was found that the β-alcohol of MeBmt was essential for cyclosporine

activity. However, the double bond has little effect, if any, on the biological activity of **1**. The major human and rabbit biliary metabolite **17** of cyclosporine was isolated by Dr. Ian Jardine (Mayo Clinic, Rochester, Minnesota) and characterized (University of Alberta). Compound **17** was found, by Dr. Jardine, to have a low biological activity which demonstrates the requirement of a hydrophobic aliphatic side chain for binding.



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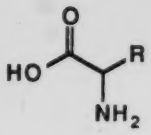
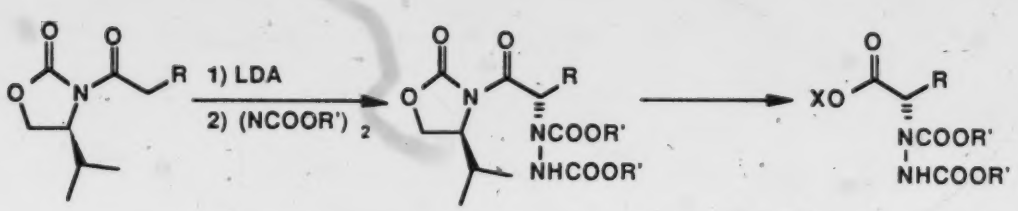


19

α-Hydrazinocarboxylic acids (**20**) are potent inhibitors of ammonia lyases and pyridoxal phosphate dependent enzymes, and possess antibiotic activity against some bacteria. In addition, these compounds can be cleaved to their parent α-amino acids **19**. A stereoselective synthesis of α-hydrazinocarboxylic acids was developed.



Enolates generated from chiral carboximides (R = CH_3, CH_2Ph, and *i*-Pr) rapidly attack dialkyl azodiformates (R' = Me, Et, CH_2Ph and *tert*-Bu) at -78 °C in THF to give aminated carboximides in 83 - 92% isolated yield. Increasing the steric bulk of either the carboximide acyl side chain or azodiformate ester improves the diastereoselectivity, with dibenzyl and di-*tert*-butyl azodiformates giving a > 90 : 10 diastereomeric ratio. Cleavage of the aminated carboximides with lithium benzyl alkoxide to the corresponding ester (X = CH_2Ph, 74-97% yield) gives epimerization in some cases (R = CH_3, CH_2Ph). However LiSH attack on the aminated carboximides followed by aqueous peracetic acid affords the corresponding acids (X = H) with little or no epimerization. Hydrogenolysis of either the benzyl ester or free acid affords **20** in yields greater than 90%. The free hydrazino acids

20 could be hydrogenolyzed to **19** by Raney nickel with complete retention of stereochemistry. The scope of this reaction was examined and a total of eight aminated carboximides were prepared.

In the final portion of this thesis, high field NMR was used to study the biosynthesis of fungal metabolites. Austin (**100**), terretonin (**104**), andilesin A (**105**), andibenin B (**106**), tajixanthone (**118**), monocerin (**119**), LL-D253α (**121**), and aspyrone (**123**) were isolated by Dr. Thomas Simpson after incorporation of precursors labelled with stable isotopes of carbon, hydrogen, and oxygen. High field NMR measurements (University of Alberta) were then done to determine the position of labelling in each of the metabolites. The development of a two-dimensional NMR technique that allows determination of which of two diastereotopic protons is deuterium labelled was also accomplished.

Acknowledgements

I am most grateful to my supervisor, Dr. John C. Vederas, for his help, support and occasional goading during my studies. Dr. Chris Bleackley, Dr. Ian Jardine, and especially Dr. Thomas Simpson are thanked for their collaborations in this work. Dr. Paul Reese and Dr. Richard N. Moore are thanked for their contributions and helpful discussions in the biosynthetic portion of this thesis. Dr. Thomas Nakashima and Mr. Glen Bigam are gratefully acknowledged for their help in obtaining high field NMR spectra. Finally, I would like to thank my wife, Belinda, whose support has helped me through these times.

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List of Abbreviations

A	antigen
Ac	CH_3CO
B	B cell
B_P	production B cell
BCDF	B cell differentiation factor
BCGF	B cell growth factor
BOC	butoxycarbonyl
Bu	butyl
CBZ	carbobenzyloxy
CI	chemical ionization
CoA	coenzyme A
DCC	1,3-dicyclohexylcarbodiimide
DMAP	dimethylaminopyridine
EI	electron impact ionization
Enz	enzyme
Et	ethyl
FAB	fast atom bombardment
FID	free induction decay
GC	gas chromatography
HPLC	high pressure liquid chromatography
IF	gamma interferon
IL-1	interleukin-1
IL-2	interleukin-2
IR	infrared spectroscopy
LDA	lithium diisopropylamide

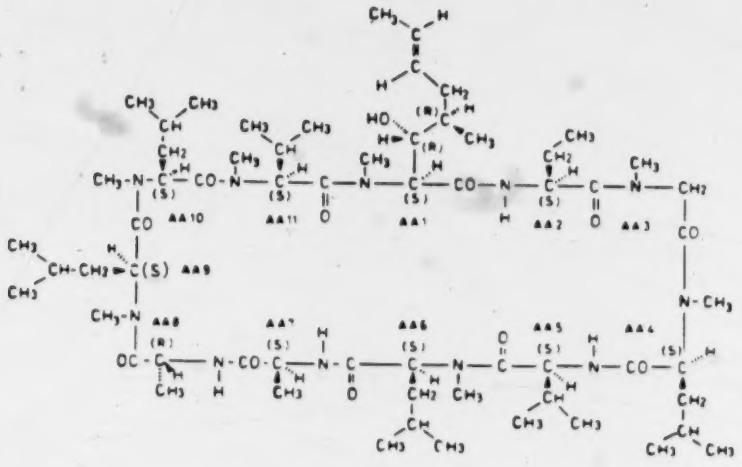
M_o	macrophage
Me	methyl
MeBmt	(4*R*)-4-[(*E*)-2-butenyl]-4,N-dimethyl L-threonine
MPLC	medium pressure liquid chromatography
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NAD^+	oxidized form of nicotinic acid adenine dinucleotide
NADPH	reduced form of nicotinic acid adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
Ph	phenyl
PLP	pyridoxal phosphate
PMA	phorbol myristate acetate
pp	pyrophosphate
ppb	parts per billion
Pr	propyl
Ra Ni	Raney nickel
SEFT	spin echo Fourier transform
T_H	helper T cell
$_pT_H$	precursor helper T cell
T_K	killer T cell
T_S	suppressor T cell
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
Ts	tosyl

Chapter 1

Studies on Cyclosporine and Its Analogs

I. Introduction

The ever-increasing number of organ transplants either from related living donors or cadavers to terminally ill patients has greatly increased the need to understand the immune response. Little change in rejection therapy had occured for two decades prior to the discovery of Cyclosporine (**1**)[1]. Since its first clinical use in 1978,[1] it has become the drug of choice in treatment of organ transplant rejection, especially those of the kidney and liver.



1

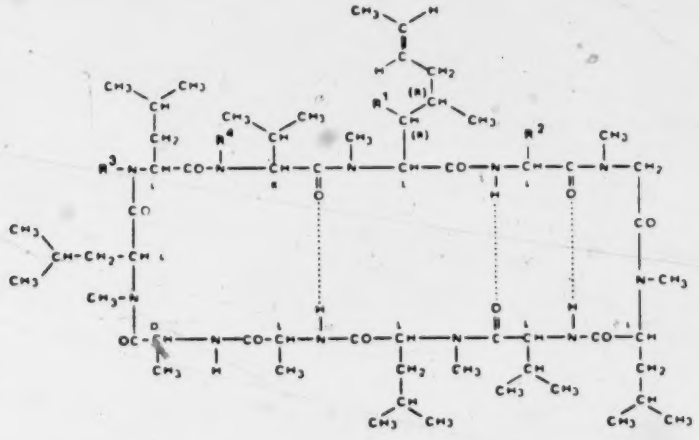
Cyclosporine, originally called "cyclosporin A", is a cyclic undecapeptide isolated from the fungal species *Tolypocladium inflatum* GAMS.[2] It contains the previously unknown amino acid (4*R*)-4-[(*E*)-2-butenyl]-4,N-dimethyl L-threonine (MeBmt) as well as several N-methylated amino acids. The compound is insoluble in water because of the highly lipophilic character of the amino acids in cyclosporine. ^{1}H and ^{13}C NMR as well as X-ray crystallography were used to determine the structure of cyclosporine.[3-6]

The unusual amino acid MeBmt has been obtained by total chemical synthesis in enantiomerically pure form from (R,R)-diethyl tartrate in a yield of 7.8% over 24 steps.[7] With MeBmt available, the Sandoz group synthesized cyclosporine very efficiently in 28%

yield with respect to this unusual amino acid.[8-10] Since the first reports of the isolation and chemistry of cyclosporine in 1976,[11] at least 8 other analogs (figure 1) have been isolated from *Tolypocladium*.[12-14]

With the knowledge that many of the analogous cyclosporins differ from the parent ~~only~~ in the amino acid at position 2, Kobel and Traber[15] undertook directed biosynthetic studies. Thus growing *T. inflatum* in the presence of L-alanine increased the amounts of cyclosporin B that could be isolated. Analogously, addition of L-threonine increased production of cyclosporin C. Growing *Tolypocladium* in the presence of D,L-α-aminobutyric acid enhanced cyclosporine (cyclosporin A) formation while simultaneously inhibiting the production of its analogs.

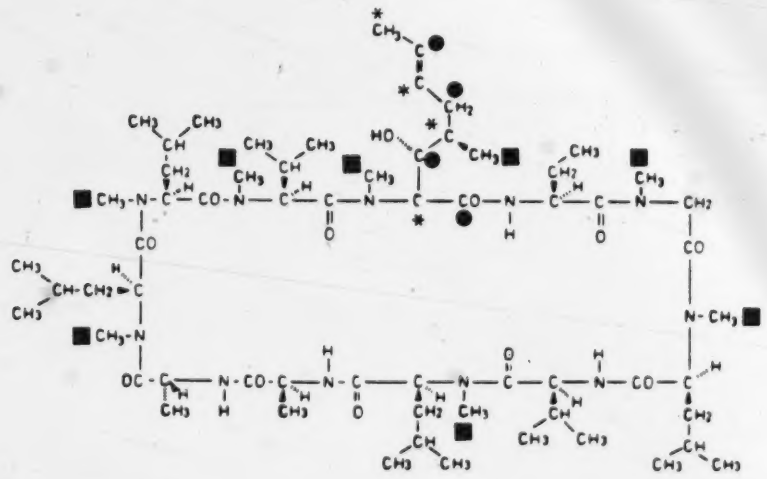
Figure 1. Structures of cyclosporins A to I.



	R^1	R^2	R^3	R^4	$R^{2)}$
Cyclosporin A	OH	CH_2CH_3	CH_3	CH_3	L
Cyclosporin B	OH	CH_3	CH_3	CH_3	L
Cyclosporin C	OH	$CH(OH)–CH_3$	CH_3	CH_3	L
Cyclosporin D	OH	$CH(CH_3)_2$	CH_3	CH_3	L
Cyclosporin E	OH	CH_2CH_3	CH_3	H	L
Cyclosporin F	H	CH_2CH_3	CH_3	CH_3	L
Cyclosporin G	OH	$CH_2CH_2CH_3$	CH_3	CH_3	L
Cyclosporin H	OH	CH_2CH_3	CH_3	CH_3	D
Cyclosporin I	OH	$CH(CH_3)_2$	H	CH_3	L

Initial biosynthetic studies by Kobel[16] using ^{13}C-labelled precursors and NMR spectroscopy indicated that the unsaturated amino acid MeBmt was assembled from head to tail coupling of four acetate units. The source of the γ-methyl and N-methyl groups was shown to be methionine using ^{3}H NMR and tritiated methionine (figure 2). Although these experiments do not indicate the site of biosynthesis, work by Zocher *et al*[17] suggested that the amino acid Bmt is synthesized at another site before incorporation into cyclosporine. The same authors also proposed that peptide assembly and N-methylation of the amino acids occur simultaneously. Partial purification of an enzyme that may be involved in the biosynthesis of cyclosporine has only recently been reported.[18] The enzyme, from *T. inflatum*, has a molecular weight of about 700 000 Daltons and was shown to catalyze the conversion of the amino acids present in the peptide chain of cyclosporine to thioesters via the corresponding adenylates. The enzyme also catalyzes N-methylation of the thioesterified amino acids present in cyclosporine.

Figure 2. Biosynthesis of cyclosporine.



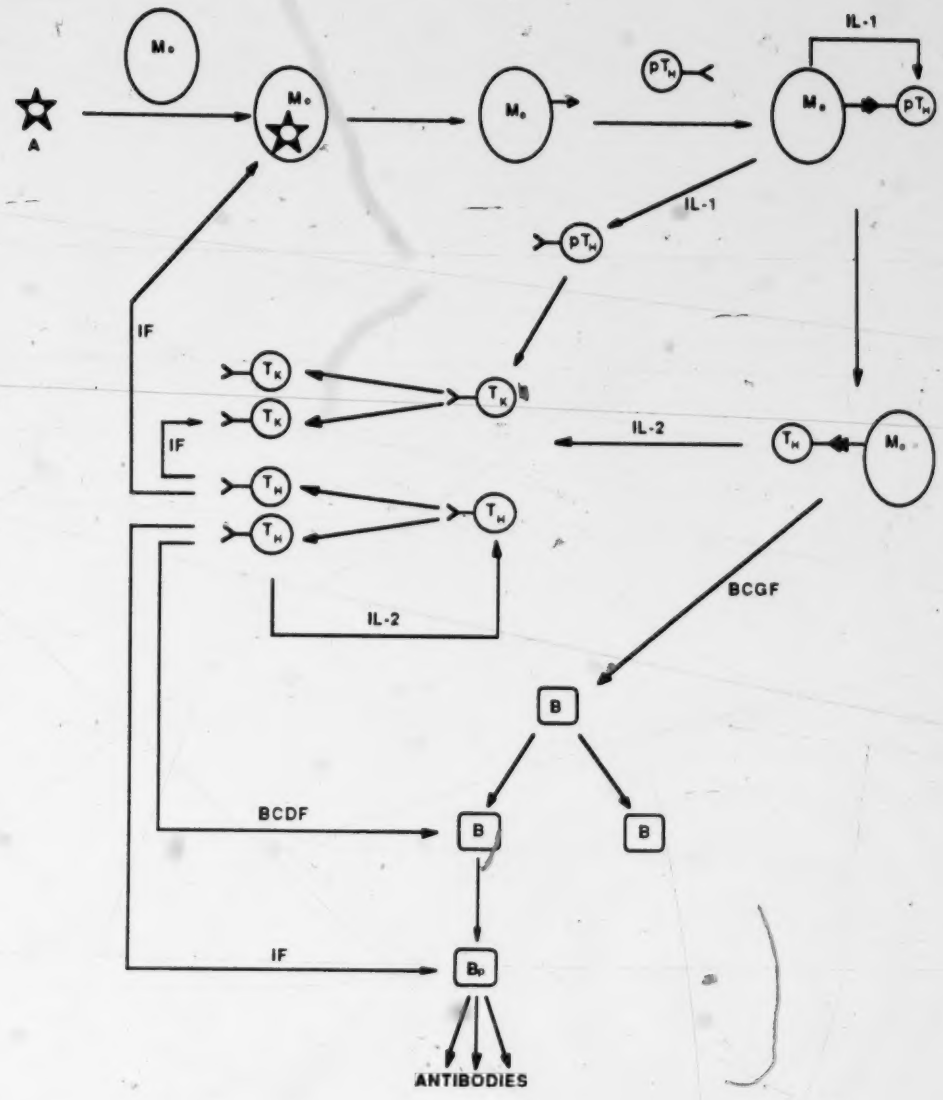
[1-^{13}C]acetate = ●

[2-^{13}C]acetate = *

[methyl-^{3}H]methionine = ■

Despite the extensive use of cyclosporine as an immunosuppressive agent, little is known about its mode of action. Understanding of the way that cyclosporine works requires a knowledge of the cellular interactions involved in immune response. The first likely defense mechanism to be encountered by an invading antigen (A) is that of the phagocytic system (figure 3), in particular a subgroup called the macrophages (M_0).[19] After ingestion of the invading organism, the macrophages (M_0), on their own surfaces, display the antigens of the invader. A precursor helper T cell (pT_H) carrying a receptor for the foreign antigen then binds to the macrophage and in doing so becomes activated. At this time the macrophage (M_0) begins to excrete the lymphokine interleukin-1 (IL-1) into the intercellular fluid. IL-1 triggers the maturation of the precursor helper T cells (pT_H) into mature helper T cells (T_H), the expression of a receptor on the T_H cell surface for another lymphokine interleukin-2 (IL-2), and the ability to secrete lymphokine IL-2. IL-2 stimulates multiplication of helper T cells (T_H) and killer T cells (T_K). Thus, production of T_H cells stimulates the multiplication of T_H cells. This serves as an efficient mechanism for rapidly increasing the T cell population and is dependent on the presence of interleukin-2 (IL-2). As mentioned previously, IL-2 also stimulates the multiplication of the few killer T cells (T_K) that are sensitive to the invading organism. The function of the T_K cells is to lyse target cells by altering membrane permeability thereby causing osmotic swelling and membrane disruption. The T_K cells survive and are able to repeat the process. The target of T_K cell activity is often an infected host cell or an invading bacterium. During the time of IL-2 production the T_H cells also produce B cell growth factor (BCGF) which stimulates the multiplication of B cells. As the number of B cells increases, the T_H cells produce B cell differentiation factor (BCDF) which causes the B cells to produce antibodies specific to the invading antigen. In addition to IL-2, BCGF, and BCDF, the T_H cells also produce lymphokine gamma interferon (IF) which activates killer T cells (T_K), thereby enabling them to better attack the invading organism. Gamma interferon (IF) also increases the ability of B cells to produce antibodies and attracts macrophages to the site of infection.

Figure 3. Schematic representation of an immune response.[19]



A = antigen
B = B cell
B_p = production B cell
BCDF = B cell differentiation factor
BCGF = B cell growth factor
IF = interferon

IL-1 = interleukin I
IL-2 = interleukin II
M_o = macrophage
pT_H = precursor helper T cells
T_H = helper T cells
T_K = killer T cells

Once the infection is controlled, suppressor T cells (T_S) halt the immune response to prevent it from spiralling out of control. Most of the T and B cells die within a few days after the infection has ended. However a group of T and B cells called memory cells are generated during the initial infection and may circulate in the blood or lymph for years, to allow the body to rapidly respond to subsequent infections.

Cyclosporine appears to inhibit the generation of the killer T lymphocytes (T_K) but does not affect the activation of suppressor T cells (T_S).[20] This imbalance between suppressor and cytotoxic cells results in transplantation tolerance. The effect of cyclosporine on B cells appears to be minimal, inhibiting only a small subpopulation of the B lymphocytes.[21] Similar inhibition patterns were also observed for the macrophages.[20] Recent work suggests that the effect on T cell population is due, in part, to a blockage in lymphokine production.[22-24] In particular, cyclosporine inhibits the secretion of interleukin 2 (IL-2) by the helper T lymphocytes (T_H).[22] This effect may be due to inhibition of calmodulin by cyclosporine.[24] Furthermore, Elliott *et al* demonstrated that the failure of production of IL-2 in both mouse tumor and human cells is caused by the inhibition of the synthesis of messenger RNA (mRNA) that codes for IL-2.[25]

Despite the wealth of knowledge that has been obtained about the effects of cyclosporine on the various cells involved in the immune response, little is known about the molecular interactions between cyclosporine and cell surface receptors or other cellular products. The goal of this project was to attempt to map out the characteristics of the "cyclosporine receptor."

II. Results and Discussion

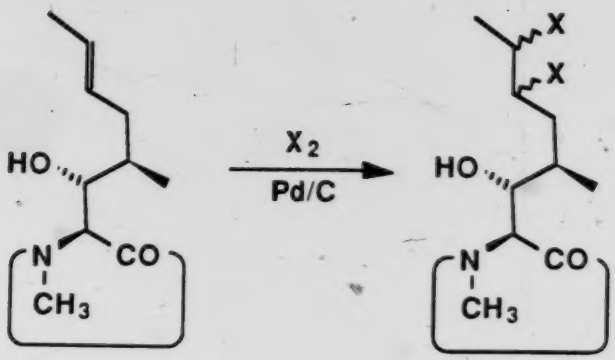
Chemical Studies

There are many options available to study the interaction of a substrate with a given receptor. In the case of cyclosporine it was decided to chemically modify its structure and study the suppression of IL-2 production in a model system. Because cyclosporine (**1**) consists of ten aliphatic amino acids and the unusual amino acid MeBmt, the sites of controllable chemical modification were limited. In particular, the peptide bonds were potential sites for modification as were the hydroxyl and vinyl moieties of MeBmt. It is conceivable that in the former case one could chemically "snip" open the peptide backbone, replace a chosen amino acid with an appropriate substitute, and then close the cyclosporine ring. However, there is no literature precedent for such an operation and it would probably be difficult to selectively cleave one of the eleven peptide bonds. In addition, analogs of cyclosporine are available from *T. inflatum* (figure 1) which contain modifications at the 2 position (cyclosporins B, C, D, G and H) or are N-demethylated at various peptide bonds (cyclosporins E and I).[12-14] These two factors discouraged modification of the backbone of cyclosporine.

The alternative, chemical alteration of MeBmt, appeared to be much more facile. Both the hydroxyl group and the double bond can act as "chemical handles" whereby modifications could be done. In addition, there were several examples in the literature in which the side chain of MeBmt had been altered.[11,14,26] The reactions of the side chain can be broken into two groups, those of the alcohol and those of the alkene.

In the latter class, the simplest reaction carried out was hydrogenation. Thus, treatment of cyclosporine (Sandoz) (**1**) with palladium on carbon under an atmosphere of hydrogen gas smoothly transformed **1** to the known[11] dihydro derivative **2** (scheme 1).

Scheme 1.



1

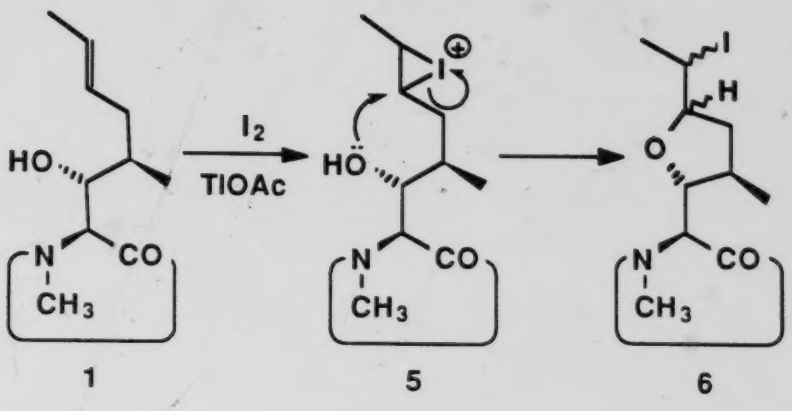
2 X = H
3 X = D
4 X = T

This method provided a simple way of introducing non-exchangable hydrogen labels (tritium or deuterium) into the molecule that could help determine of the site of binding of **1** in biological systems.

To determine the specificity of labelling, **1** was converted in 98% yield to its dideutero derivative **3** (scheme 1). However, the tritiation to form ditritio derivative **4** was carried out at Amersham laboratories because of the difficulty in handling tritium gas having high activity. Using conditions identical to those described for the production of **2** and **3**, 19 mg of cyclosporine (**1**) was hydrogenated (scheme1) with tritium gas at Amersham. After purification, 17 mg of [$^3H_2$]cyclosporine (**4**) was obtained with an activity of approximately 40 Ci/mmol. This material decomposed rapidly by autoradiolysis.

Treatment of cyclosporine (**1**) with iodine and thallium acetate using the procedure of Cambie *et al* [27,28] did not give the iodoacetate.[11] Instead the known[11] cyclic ether **6** was obtained (scheme 2). Presumably this product arises from attack of the

Scheme 2.

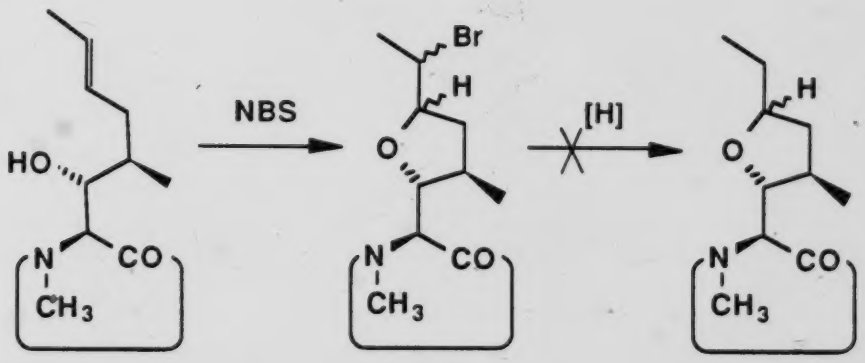


1 5 6

β-alcohol on the iodonium cation intermediate **5**. The reaction of **1** with thallium acetate and iodine in chloroform afforded **6** in 58% yield.

The bromo analog **7** was formed analogously. Here the goal was to produce the dehalogenated cyclic ether **8** by reductive treatment of the bromide **7** as depicted in scheme 3. Thus, reaction of N-bromosuccinimide with **1** using the conditions of Demole *et al* [29]

Scheme 3.

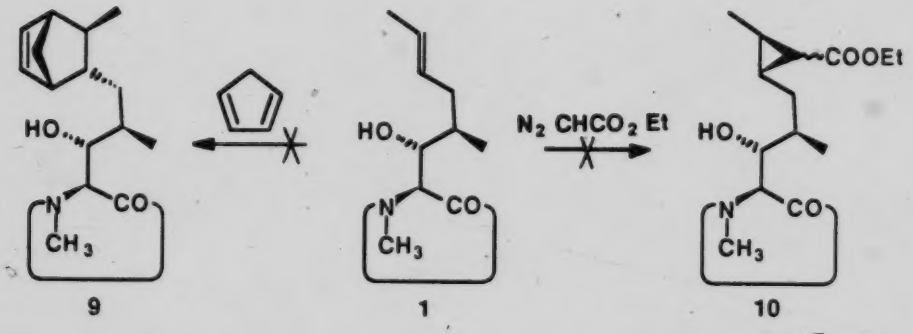


1 7 8

gave the cyclic ether **7** in 40% yield. However, attempts to cleave the carbon-bromine bond using either catalytic hydrogenolysis[30] or treatment with tributyltin hydride[31] failed.

Attachment of a fluorescent label to cyclosporine (**1**) via reaction with the double bond would also provide a marker to identify the site of the cyclosporine receptor. However, this would only be the case if the modified analog does indeed bind to the receptor. Model studies using reactive precursors to which a fluorescent label could be attached were done. The first of the two models employed cyclopentadiene which is a potent diene for Diels Alder reactions. This compound was of interest because it could potentially be converted to its carbanion with strong base and alkylated with a fluorescent agent. This would give a fluorescent diene which might undergo electrocyclic addition to the double bond of the MeBmt residue. Unfortunately after reaction of a neat solution of cyclopentadiene with **1** no trace of the adduct **9** could be found (scheme 4). Only cyclosporine (**1**) and cyclopentadiene dimer were isolated. This indicated that a more hindered fluorescent diene would also fail to add to the double bond. For this reason the model studies with cyclopentadiene were not continued.

Scheme 4.



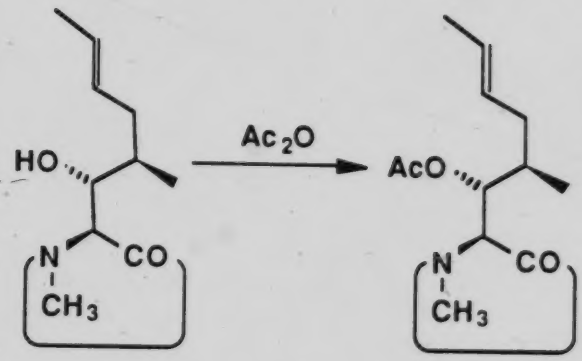
The other model employed ethyl diazoacetate.[32] This compound was of interest because a fluorescent label could potentially be introduced via the ester functionality.

Copper mediated decomposition of the diazo ester[33] might then allow carbenoid type addition to the double bond of cyclosporine (**1**) (scheme 4) to afford the cyclopropane **10**.[34,35]

Treatment of cyclosporine with a mixture of ethyl diazoacetate and soluble copper salts gave only recovered **1**, diethyl maleate and diethyl fumarate. The latter two compounds are the cis and trans dimer products, respectively, of the carbene and are usually the major products unless the alkene acts as the solvent. As a result, this route was also abandoned.

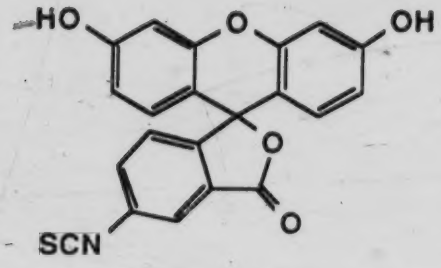
In the second class of reactions of cyclosporine, those involving functionalization of the β-alcohol, acetylation is the simplest (scheme 5). The known[14] β-acyloxy

Scheme 5.



1 **11**

compound **11** was obtained in excellent yield by reaction of acetic anhydride and dimethylaminopyridine with **1**. In a final attempt to introduce a fluorescent label into cyclosporine, fluorescein **12** and dansyl chloride **13**[36], both of which are known to react with free alcohols, were mixed with cyclosporine. Neither compound gave an adduct with **1**.



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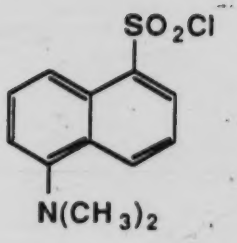
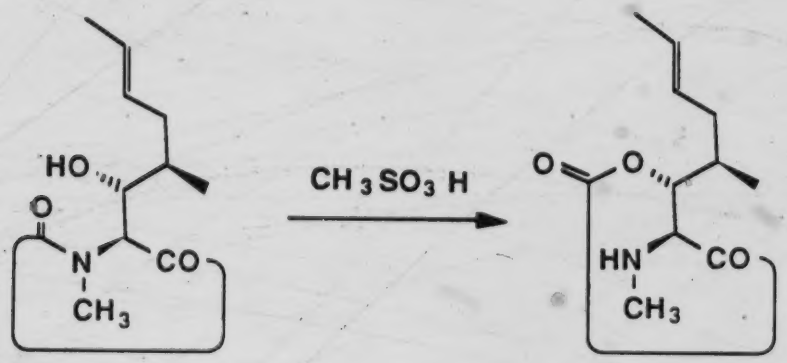


13

The final analog of cyclosporine prepared was the known compound isocyclosporine[11] (**14**). Reaction of cyclosporine (**1**) with methanesulfonic acid in

Scheme 6.



1 14

methanol (scheme 6) resulted in a N, O-acyl migration of the N-methyl valine carbonyl group at position 11 in 51% yield. This produced a new ester linkage and a free N-methyl amino group.

The lack of reactivity of the double bond and alcohol might be explained in part by the conformation of cyclosporine. The x-ray structure of cyclosporine[6] (figure 4a) indicates that in the solid state the side chain of MeBmt is folded into a cleft formed by the methyl leucines at positions 4 and 6. As expected, the solution conformation is somewhat less rigid. Loosli *et al*[6] used NMR and IR to show that there is surprisingly little difference

in backbone conformation between the solid and solution states. Increased mobility is observed in the side chain of MeBmt once dissolved (figure 4b).

Despite the ability of the side chain to swing out of the cleft, the hydroxyl function must remain in a sterically congested area. This suggests that only small electrophiles such as acetic anhydride may approach it while larger molecules such as **12** and **13** are unable to gain access.

Figure 4. Solid state and solution conformations of cyclosporine.

The conformation of the far end of MeBmt remains unclear. Presumably the olefin is somewhat removed from the crowded backbone. Because of this, cyclosporine can approach a palladium surface and allow catalytic hydrogenation to occur. However, once the cyclic ether **6** or **7** is formed, the iodine or bromine would be pulled back into the crowded cleft of cyclosporine. This may explain the inability to dehalogenate the ether **7**. In the case of cyclopentadiene, the failure to form Diels-Alder adducts with cyclosporine

(**1**) may be explained in terms of electronic effects. Although cyclopentadiene is a highly reactive diene, it is unlikely that the olefin of MeBmt is sufficiently electron deficient to readily undergo electrocyclic reaction.[37]

Biological studies

The model systems used to study the suppression of interleukin 2 production were the subclone EL4.E1 of the mouse T lymphoma EL4 line and the subclone JEH.3B of the human T leukemia line Jurkat. These studies were done by Chris Bleackley (Department of Biochemistry, University of Alberta, Edmonton, Alberta). The above cell strains were chosen because of their high levels of IL-2 secretion upon chemical stimulation with phorbol myristate acetate (PMA). EL4 and Jurkat cells were stimulated with PMA for 24 hours in the presence of varying amounts of cyclosporine or its analogs. The relative IL-2 concentrations in the cell culture supernatents were then measured using a literature

Table 1. Level of cyclosporine analog to produce 50% suppression of IL-2 production.

Compound	Name	Concentration (ng/mL)
1	cyclosporine	10
2	dihydrocyclosporin	20
3	dideuterocyclosporin	20
6	iodocyclosporin	no activity
7	bromocyclosporin	no activity
11	O-acetylcyclosporin	300
14	isocyclosporin	10

assay[38] which employed an IL-2 dependent cell line. The concentration of cyclosporine or its analog required to reduce the IL-2 concentrations by 50% in either the EL4 or Jurkat cell lines is shown in table 1.

From these results it is clear that the unsaturated amino acid MeBmt is essential for cyclosporine's activity and that even small chemical modifications have a profound effect on its immunosuppressive properties. It appears that the double bond plays little or no role in the binding of **1** to its receptor. This is indicated by the fact that hydrogenation to give a saturated side chain does not significantly decrease the biological activity.

However, it is evident that the β-alcohol of MeBmt is essential for biological activity. Once this moiety is masked by acetylation as in **11**, the activity of the analog is reduced thirty fold. Similarly, in the cases where the OH is contained in a cyclic ether, no activity is observed.

Incubation of isocyclosporin (**14**) with EL4 and Jurkat cells gave a surprising result. Despite the masking of the β-alcohol by the newly formed ester, this derivative was found to be as active as cyclosporine itself. There are two possible explanations for this observation. For an analog of cyclosporine to be active it may not need to have an alcohol at the β-position but could instead have another polar group in its place. Thus, isocyclosporin activity may be due to the N-methyl amino group occupying a position similar to that of the alcohol in **1**. A second and perhaps more reasonable explanation is that within the cell, isocyclosporin is in equilibrium with cyclosporine, and it is the latter of the two whose effect is being observed. This is not without literature precedent since it was demonstrated that **1** and **14** can equilibrate *in vitro* under certain conditions.[11]

These results are in good agreement with those published recently[10,39] and provide structure-activity relationships for **1** and its analogs. However much work remains to be done before a more exact picture of the binding site can be obtained.

NMR and Metabolism Studies

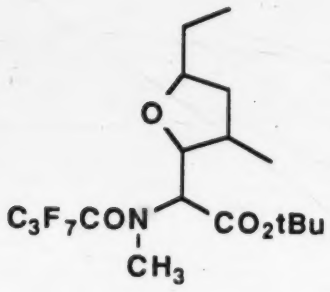
Despite the effectiveness of cyclosporine as an immunosuppressive agent, it possess many drawbacks. These include serious side effects such as nephrotoxicity, gum hypertrophy, hirsutism, tremor and depressive psychosis.[40] Nephrotoxicity is usually the most severe problem. Since high pressure liquid chromatography (HPLC) consistently gives lower levels of cyclosporine than radioimmunoassay, the presence of structurally similar metabolites of **1** is indicated.[41] Whether the toxic side effects are due to **1** or the metabolic products of **1** is not known.

Recently Mauer *et al*[42] described several hydroxylated and N-demethylated metabolites from rats, dogs and humans. In collaboration with Ian Jardine (Department of Pharmacology, Mayo Clinic, Rochester, Minnesota) the primary biliary metabolite in rabbit and human bile was isolated (Mayo Clinic) and characterized (University of Alberta).[41]

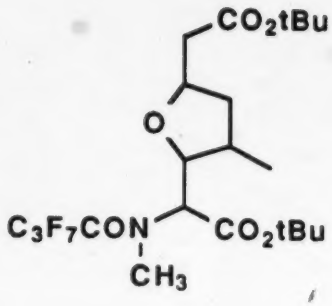
Fast atom bombardment mass spectrometry (FAB MS) of the parent cyclosporine (**1**) gave two peaks of mass 1203 and 1090 corresponding to the protonated molecular ion (MH^+) and loss of the side chain of MeBmt respectively. FAB MS of the major biliary metabolite gave peaks at 1233 and 1090. The increase of thirty mass units was interpreted as the conversion of a methyl group to a carboxylic acid. The second peak at 1090 mass units suggests that the conversion occurred at the 1 position of the cyclosporine.

To determine exactly which of the amino acids was modified, the metabolite was hydrolysed with HCl and the resulting amino acids were esterified with isobutanol/HCl, acetylated with heptafluorobutyric anhydride, and analysed by combined gas chromatography/mass spectrometry (GC/MS). Under these conditions MeBmt is known[11] to cyclize via acid catalyzed attack of the alcohol on the alkene to produce cyclic ethers. In the case of the metabolite, no GC peak corresponding to **15** was observed. Instead, a new peak appeared. The electron impact mass spectrum of this new peak was similar to that of **15**, and suggested the presence of a *t*-butyl ester. The fragmentation pattern indicated that

the oxidation had occurred at the terminus of the side chain of MeBmt to give the derivatized structure **16**.



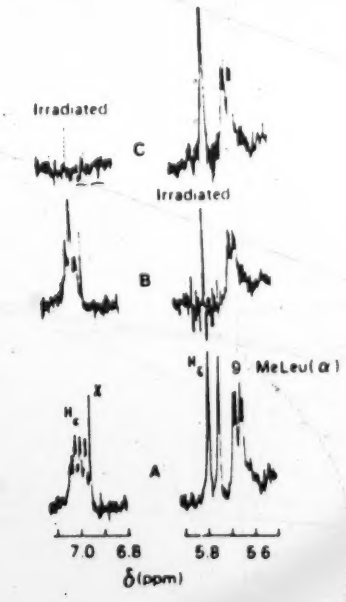
15



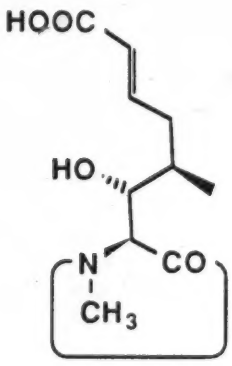
16

Thus, it appears that the major biliary metabolite of cyclosporine is a carboxylic acid derivative. However, it remained unclear whether the cyclization of the alcohol occurred in the biological system or during acid digestion. To answer this problem ^1H NMR spectroscopy was employed. Expansions of the regions from 7.1 - 6.8 and 5.9 - 5.5 ppm of the normal proton spectrum of the metabolite are shown in figure 5a. The multiplet at

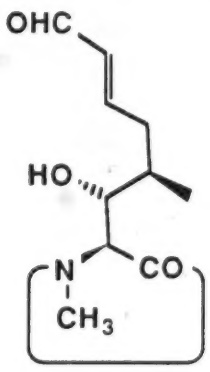
Figure 5. Partial ^1H NMR spectrum of major cyclosporine metabolite



7.01 ppm and the doublet (J = 15.6 Hz) at 5.79 ppm are not in the corresponding cyclosporine spectrum and each integrates for 1 proton. The doublet of doublets at 5.67 ppm has been previously assigned to the α-proton of methyl-leucine at the 9 position.[4] Irradiation of the doublet (figure 5b) causes collapse of the multiplet at 7.01 ppm to a triplet. In the complementary experiment in which the multiplet at 7.01 ppm was irradiated, the doublet collapsed to a singlet (figure 5c). The peak marked X is assigned to the upfield ^{13}C satellite of $CHCl_3$. These data are indicative of a trans α,β-unsaturated carboxylic acid, and when taken together with the mass spectral results, identify the primary biliary metabolite of **1** as the α,β-unsaturated acid **17**.



17



18

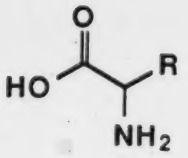
It is interesting to note that the major metabolite showed no immunosuppressive activity.[41] This observation implies that the hydrophobic aliphatic side chain terminus of MeBmt is essential for activity. It is possible that this metabolite and its probable precursor **18** may contribute to the toxicity of cyclosporine through Michael additions with nucleophilic cellular constituents.

Chapter 2

Asymmetric Synthesis of α-Hydrazino Acids and α-Amino Acids

I. Introduction

Since α-amino acids (**19**) are potential intermediates in enantioselective synthesis,[43-46] and since there are over 700 naturally occurring amino acids,[47] much recent work has been published on the asymmetric synthesis of these compounds.[48-53]



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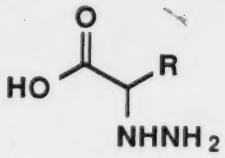
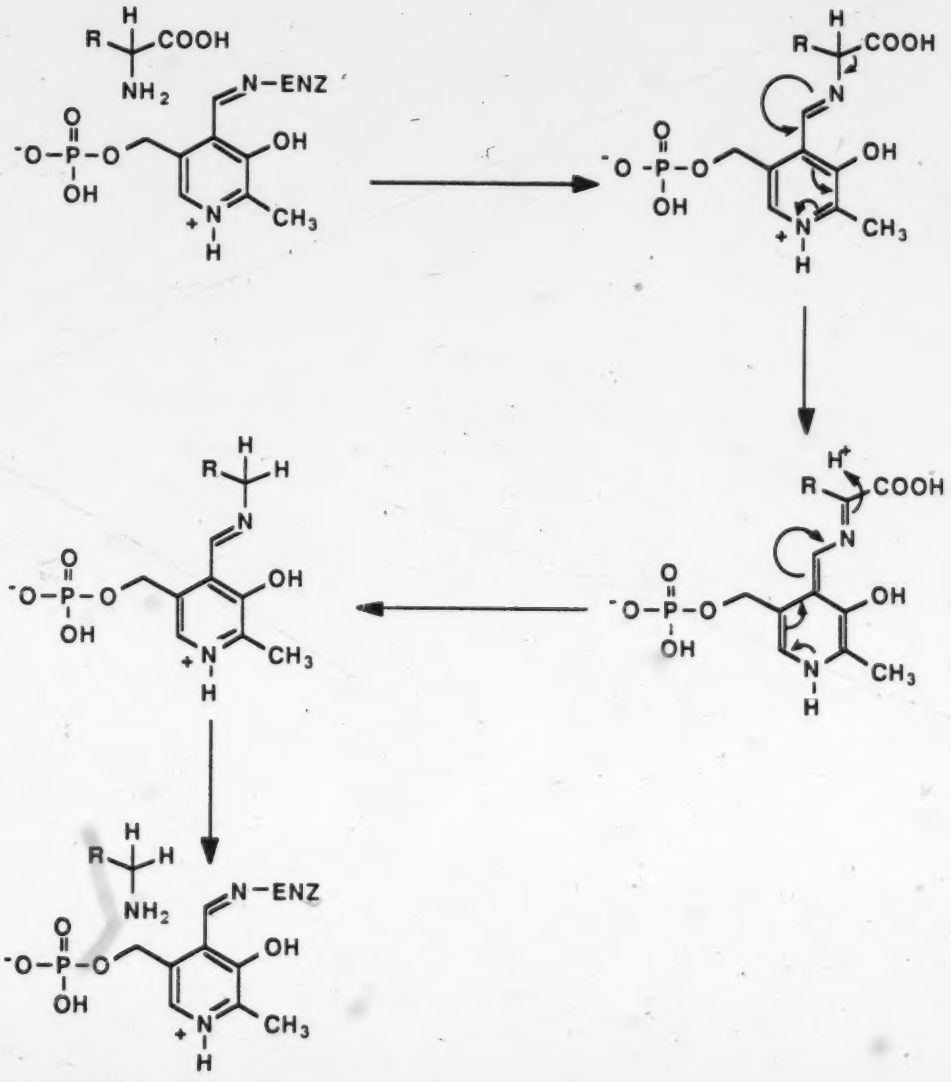


20

The structurally related α-hydrazinocarboxylic acids (**20**) are a class of compounds that are known as strong competitive inhibitors of enzymes that metabolise amino acids. These include ammonia lyases[54,55] and pyridoxal phosphate (PLP) dependent enzymes such as: histidine decarboxylase;[56-58] aspartate aminotransferase;[59,60] ornithine decarboxylase;[61-64] ornithine-keto acid aminotransferase;[62] diaminopimelate decarboxylase;[65] dopa decarboxylase[66] and aromatic amino acid decarboxylase.[67]

Because of these properties, α-hydrazino acids often possess antibiotic activity against a variety of bacteria such as *Azotobacter* sp. 211,[70] *Escherichia coli*,[68-70] *Pseudomonas fluoescens*,[70] *Corynebacterium xerosis*,[70] *Bacillus megaterium*,[65,70] *Bacillus subtilis* [65,70] and *Salmonella dublin*.[69] In addition to the antibiotic properties of α-hydrazinocarboxylic acids, several interesting physiological effects have also been observed. These include: reduction of renal histamine concentration in diabetic rats by α-hydrazino histidine;[71] inhibition of ethylene production in mung beans and apples by α-hydrazino-β-phenylpropionic acid;[72] inhibition of egg cleavage in fertilized sea urchin eggs by α-hydrazino ornithine,[73] and generation of a relatively specific histidine ammonia-lyase inhibition in rats.[74]

Figure 6. Mechanism of pyridoxal phosphate dependent decarboxylation.



The mechanism of PLP dependent enzyme catalysis is well understood[75-79] and is illustrated in figure 6 for the decarboxylases. Pyridoxal phosphate is found in the active site covalently bound to the enzyme via an imine bond with the ε-amino group of a lysine residue.[75] Once the substrate has entered the active site, transimination occurs between the α-amino group of the substrate and the lysine residue.[79] Decarboxylation is then facilitated by the electron deficient protonated pyridine ring. This ring acts as a site to store the electrons of the cleaved bond. In the case of epimerases and transaminases, the reaction proceeds analogously but instead of CO_2 release the α-proton is lost.

The intermediate is then protonated, usually with retention of stereochemistry (i.e. the new hydrogen occupies the same position as the substrate carboxyl group).[75-78] However, in some rare cases the decarboxylation has been shown to proceed with inversion of stereochemistry.[79-81] Finally, either transimination or hydrolysis releases the product and regenerates the pyridoxal phosphate cofactor.

Based on the above mechanism, it is possible to explain enzyme inactivation with α-hydrazinocarboxylic acids. Presumably once inside the active site, compounds such as **20** can form a hydrazone via transimination as depicted in figure 7 . This complex blocks

Figure 7. Possible mechanism of inhibition of pyridoxal phosphate dependent enzymes by α-hydrazinocarboxylic acids.

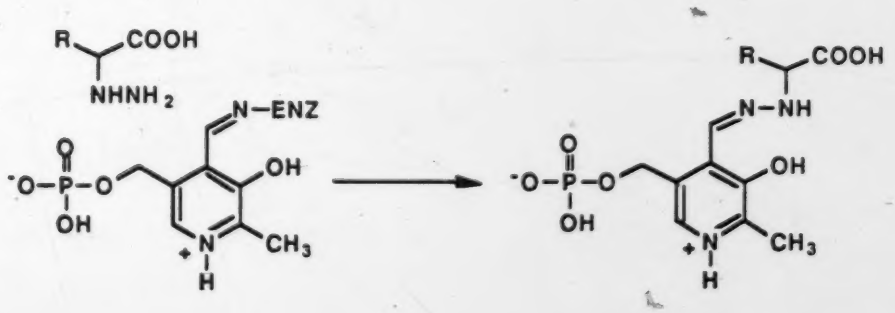
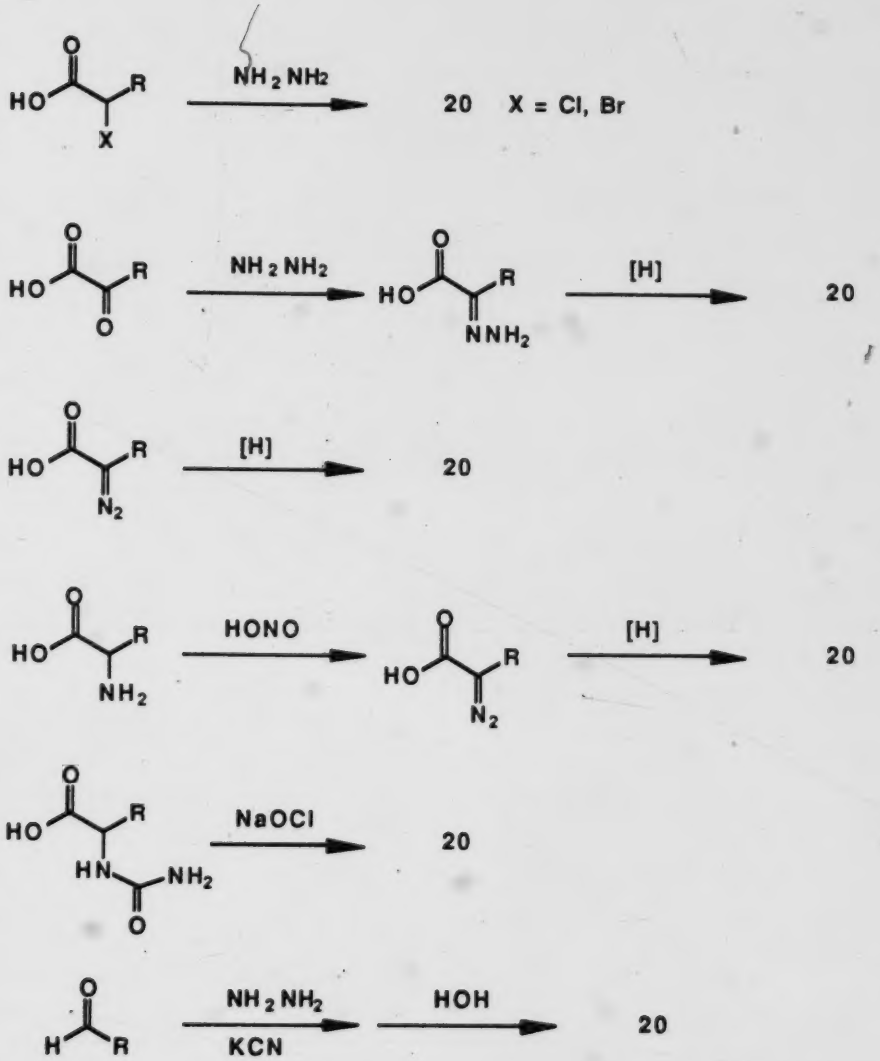


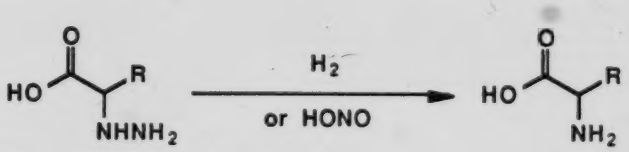
Figure 8. Methodologies for the synthesis of α-hydrazinocarboxylic acids.



the enzyme from accomplishing its catalytic task. The hydrazone is likely to remain bound to the cofactor since it is much less susceptible to hydrolysis than an ordinary aldimine intermediate.

Several methods for the synthesis of compounds **20** have been reported and are shown in figure 8. The most commonly used method for the preparation of **20** involves the treatment of α-halo acids with hydrazine.[82-86] Other less common procedures include the reduction of the hydrazones of α-keto acids,[66,87] reduction of α-diazo esters,[88] nitrosation-reduction of α-amino acids,[89] Hofmann rearrangement of α-ureido acids,[90-92] and reaction of aldehydes with hydrazine and cyanide in a Strecker approach.[93,94] The utility of these methods is often diminished because of loss of optical purity or low yields.

Figure 9. Conversion of α-hydrazino acids to α-amino acids.



20 **19**

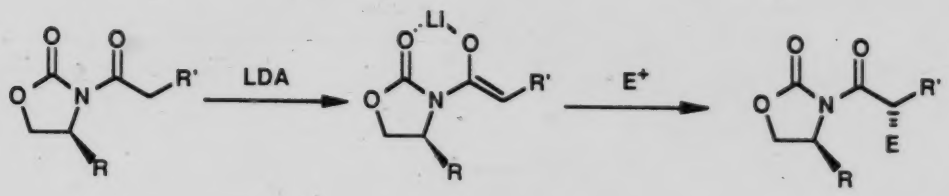
α-Hydrazinocarboxylic acids (**20**) are known to be cleaved to their parent α-amino acids (**19**) either by catalytic hydrogenolysis of the nitrogen-nitrogen bond[92] or by nitrosation[90] (figure 9). Thus a convenient synthesis of α-hydrazinocarboxylic acids (**20**) also provides a simple route to α-amino acids (**19**). These attributes make an efficient stereospecific synthesis of α-hydrazinocarboxylic acids highly desirable.

II. Results and Discussion

Several problems must be considered when designing an asymmetric synthesis of amino acids and amino acid analogs. Initially the aim was to convert protected carboxylic acids into amino acids. This could be done in one of two ways, by direct amination of an enolate bearing the appropriately substituted side chain or by alkylation of a protected glycine enolate. The problems involved in the introduction of an asymmetric center at the α-position must also be overcome.

Recent studies by Evans and coworkers[95-100] have demonstrated that the enolates of chiral carboximides shown in figure 10 react with electrophiles in a highly

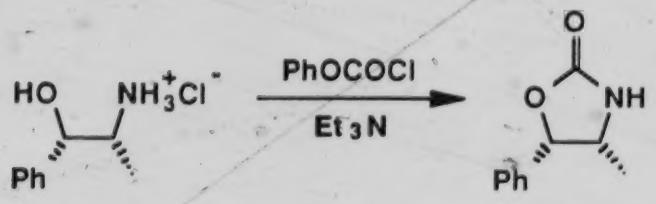
Figure 10. Proposed mechanism for the addition of electrophiles to chiral enolates.



diastereoselective fashion to give predominantly one of the two possible diastereomeric products. Evans proposes that lithium diisopropylamide (LDA) treatment of the carboximide forms an enolate that exists only in the Z form due to steric repulsion between the side chain R' and the oxazolidinone. Rotation about the nitrogen-carbon bond is inhibited because chelation of the lithium cation with the enolate oxygen and the carbonyl of the oxazolidinone holds the molecule in one conformation. Finally, approach of an electrophile from the *re* face (as drawn with R' = alkyl) is prohibited because the large R group hinders that side. Consequently electrophilic attack occurs primarily from the *si* face.

The electrophiles studied to date have been: aldehydes;[100] alkyl halides;[99] acid chlorides[98] and oxygen donors.[97] Since chiral alkylations work well in this system[98] and since recent work had demonstrated the feasibility of alkylation of glycine derivatives,[50] it seemed reasonable to combine these methods and prepare optically active amino acids by alkylation of chiral glycine derived enolates.

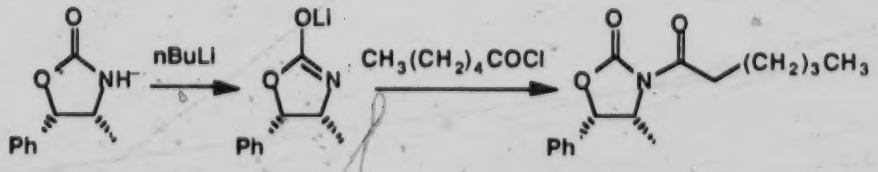
Scheme 7.



21 22

In a series of model studies chiral oxazolidinone **22**[100] was used. Preparation of this compound involved treatment of commercially available (1*S*,2*R*)-norephedrine hydrochloride (**21**) with phenyl chloroformate (scheme 7). Treatment of the lithium salt **23** of **22** with hexanoyl chloride gave the chiral carboximide **24** (scheme 8). Methyl

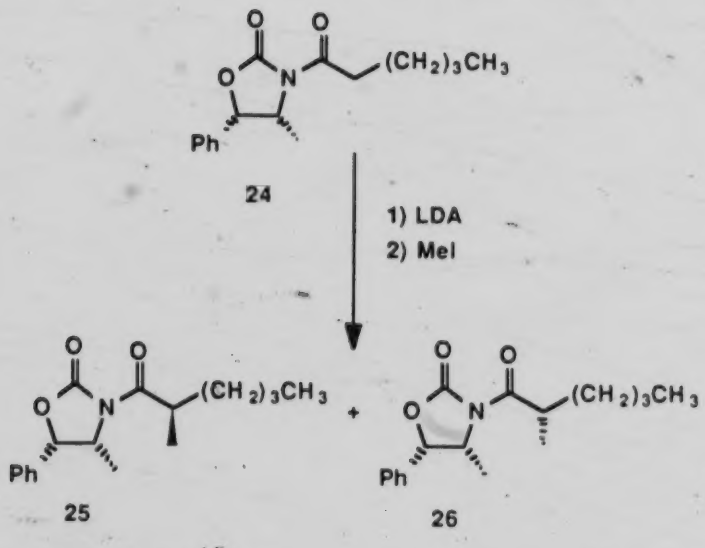
Scheme 8.



22 23 24

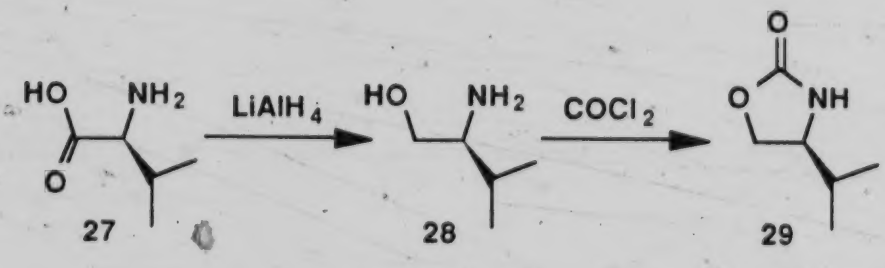
iodide quenching of the enolate of **24** formed by LDA afforded the alkylated product **25** (scheme 9). Compound **25** was free of contamination by the unwanted diastereomer **26** after purification by column chromatography. However, large scale alkylation of **24** not only gave **25** but also appreciable amounts of the unwanted diastereomer **26** (scheme 9).

Scheme 9.

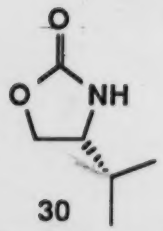


To circumvent this problem, the use of the oxazolidinone **22** was discontinued. It appeared that a larger directing group could possibly increase diastereoselection. Preparation of the chiral (*S*)-oxazolidinone **29**[96,101] was accomplished by phosgene treatment of L-valinol (**28**) (scheme 10). Compound **28** was obtained by lithium

Scheme 10.



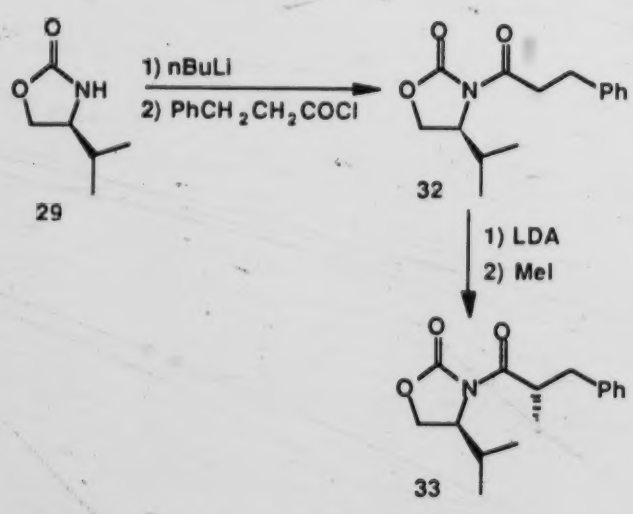
aluminum hydride reduction[102] of L-valine (**27**). The (*R*)-oxazolidinone **30** was prepared in a similar fashion from D-valinol (**31**).



30

Acylation of the lithium salt of **29** with dihydrocinnamoyl chloride gave the known[96] carboximide **32** (scheme 11). Sequential treatement of **32** with LDA and methyl iodide afforded the alkylated product **33** in 77% yield. The ^{1}H NMR spectrum of compound **33** showed no trace of the unwanted diastereomer after purification by chromatography.

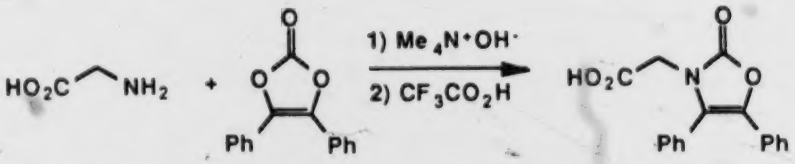
Scheme 11.



As a result of the success of the above experiments, the synthesis of a "glycinated" oxazolidinone was undertaken. In order to form the acid chloride of glycine (**34**) the amine must first be protected. Reaction of **34** with cyclic carbonate **35** using the method of Sheehan *et al* gave the known oxazolone **36**[103] (scheme 12).

Compound **36** was converted to its acid chloride **37** with thionyl chloride (scheme 13). Treatment of **37** with a solution of the lithium salt of **29** gave the desired carboximide **38** in a yield of 40%.

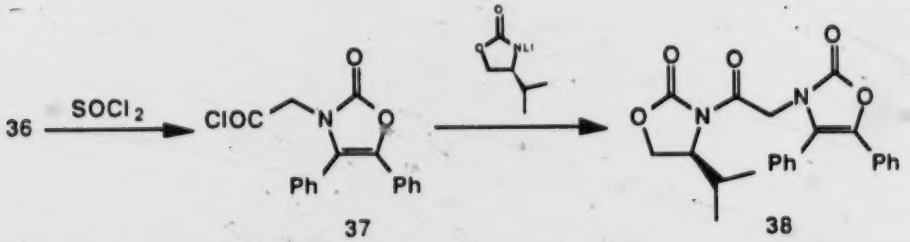
Scheme 12.



34 35 36

It was hoped that carboximide **38** would serve as a source for the generation of a chiral glycine enolate, and that this enolate could then be alkylated with methyl iodide

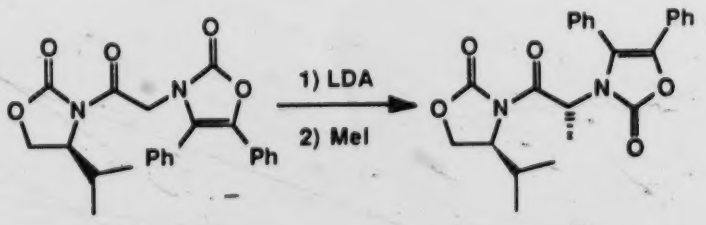
Scheme 13.



37 38

to give the protected alanine derivative **39** in a diastereoselective manner (scheme 14). However, all attempts to alkylate the lithium enolate of **38** with methyl iodide, or other electrophiles such as propyl iodide or benzyl bromide, failed.

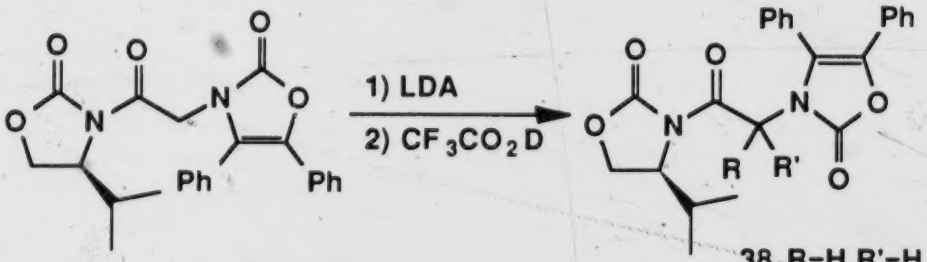
Scheme 14.



38 39

In an effort to determine whether or not the enolate was formed, **38** was treated with LDA and quenched by the addition of trifluoroacetic acid-d_1 (scheme 15). Recovery of the product was nearly quantitative. Mass spectroscopy showed that the material was 38% deuterated (**40**) and 22% dideuterated (**41**) at the α-position. This indicates a high level of enolate formation. Treatment of **38** with trifluoroacetic acid-d_1 failed to introduce deuterium into the α-position. Careful study of the normal proton NMR showed that both doublets of the AB pattern due to the α-protons had equal peak areas. Thus, the protonation/deuteration is non diastereoselective. Under these conditions it appears that carboximide **38** cannot serve as a precursor to chiral isotopically labelled glycine.

Scheme 15.



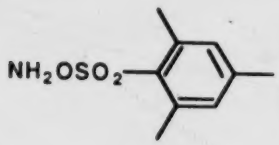
38

38 R=H,R'=H
40 R=H,R'=D
41 R=D,R'=D

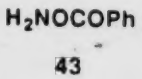
Since deuteration demonstrated that the enolate was indeed being formed, the cause for the lack of alkylation must lie elsewhere. Examination of molecular models of **38** shows that the α-position is extremely hindered by both the isopropyl and phenyl groups of the oxazolidinone and oxazolone.

The inability to alkylate the enolate of **38** made the alternative approach, amination of enolates, much more attractive. A convenient source of electrophilic nitrogen would permit direct introduction of this atom at the α-position. Currently, most sources of electrophilic nitrogen[104-117] are composed of an amine bound to an electronegative leaving group. The most common of these are derivatives of hydroxylamine such as O-mesitylenesulfonylhydroxylamine (**42**), O-benzoyl hydroxylamine (**43**), O-(p-

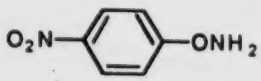
nitrophenyl)-hydroxylamine (**44**) and O-methylhydroxylamine (**45**). However, chloramine (**46**) and tosylazide (**47**) are also commonly used for aminations[104]. All of these reagents are readily available and smoothly aminate such nucleophiles as amines, sulfides, sulfoxides, and phosphines in good to very good yields.



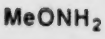
42



43



44



45

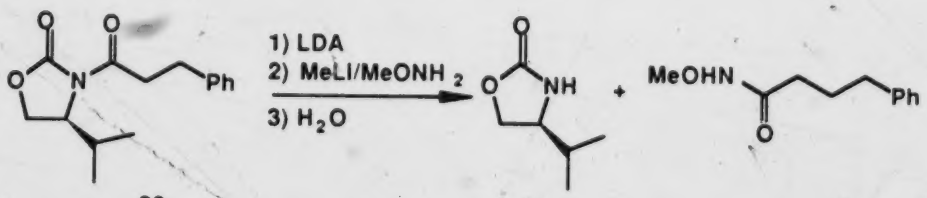
46

47

Unfortunately, the yields obtained when these compounds react with carbon nucleophiles are often disappointing. However, recent work has demonstrated that sulfur activated azides readily attack Grignard reagents[109,110] and that methoxylamine-methyllithium mixtures aminate organolithium compounds[111,112] in high yields.

Treatment of the enolate of **32** with a mixture of methoxylamine and methyllithium using the procedure of Beak[111] (scheme 16) followed by aqueous workup did not afford the expected aminated product but instead gave the cleavage products **29** and **48**.

Scheme 16.

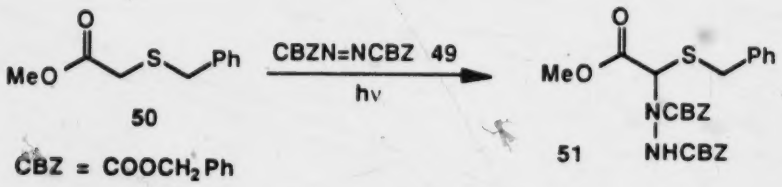


32 **29** **48**

In the light of these results a second class of potential aminating agents, the dialkyl azodiformates, was studied. Work by Stolle *et al*[118] demonstrated that Grignard reagents

added to N-acyl azo compounds in modest yield. The condensation of Grignard reagents with dialkyl azodiformates was shown to be a synthetically useful method by the group of Carpino.[119] Enamines have been added successfully to the closely related N-phenyl triazolinedione.[120] Although several other nucleophiles attack dialkyl azodiformates,[121] there were no reports of the nucleophilic addition of enolates to these compounds. The somewhat analogous reaction of dimethyl and dibenzyl (**49**) azodiformates with methyl S-benzylthioglycollate (**50**) is an ene type reaction[122] (scheme 17).

Scheme 17.

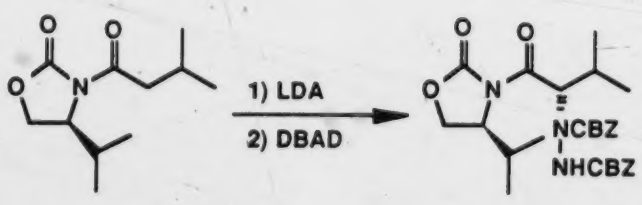


50 **51**

CBZ = $COOCH_2Ph$

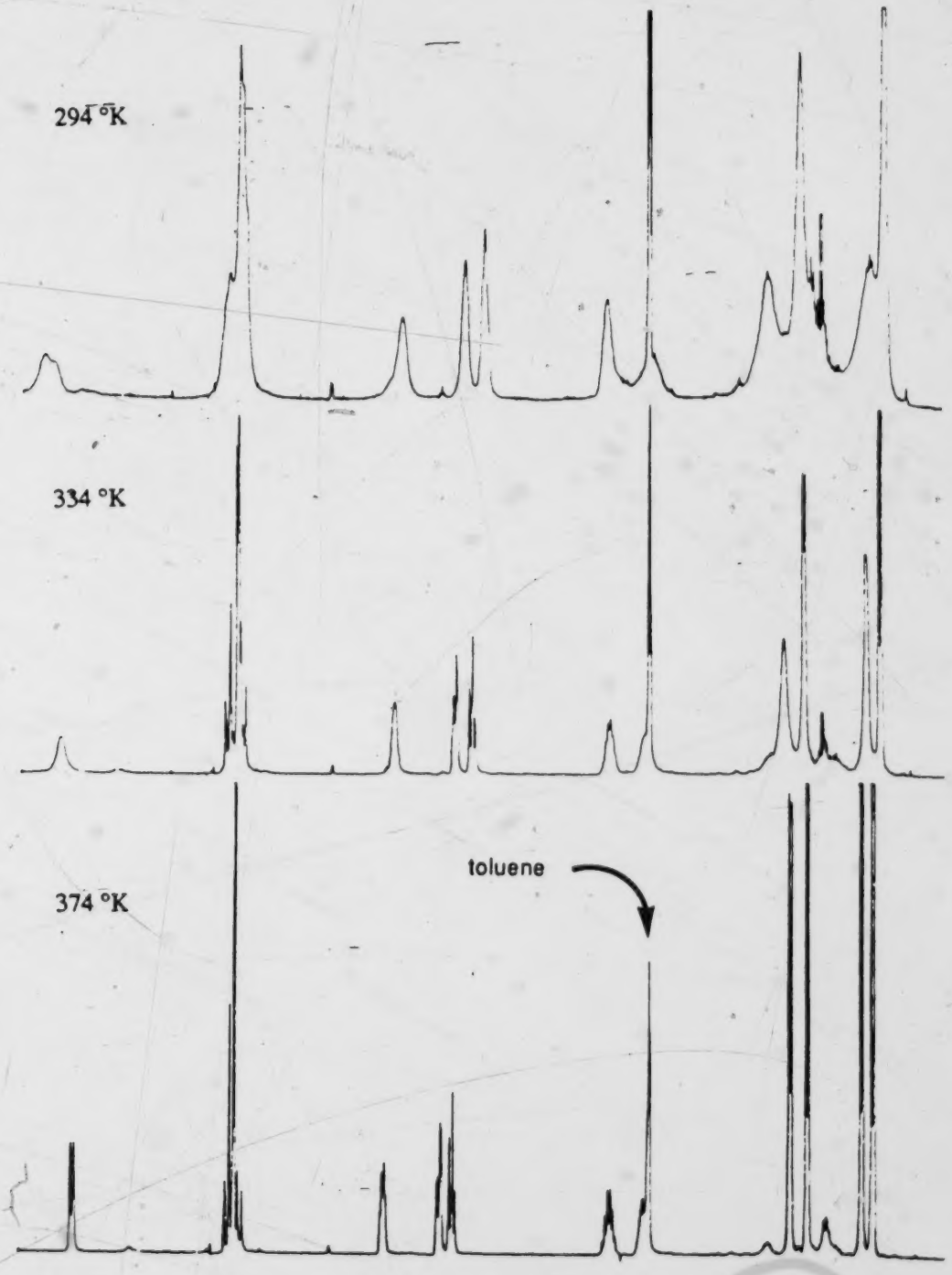
In a test case the carboximide **52** formed from oxazolidinone **29** and *iso*-valeryl chloride was converted to its lithium enolate at -78 °C in THF. The anion was quenched at -78 °C with commercially available (TCI Organics) dibenzyl azodiformate (DBAD) (**49**). Only one compound was isolated in high yield whose IR, MS, and analysis were consistent with the proposed structure **53** (scheme 18). However the normal ^1H NMR gave exceedingly broad peaks whose positions corresponded with the chemical shifts expected for **53**.

Scheme 18.

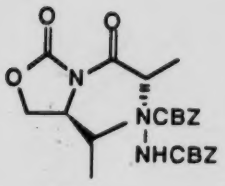


52 **53**

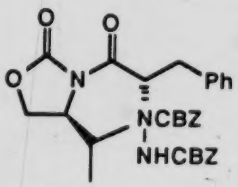
Figure 11. Variable temperature ^1H NMR spectra of aminated carboximide **53** in toluene-d_8. The chemical shift range is from 6.50 ppm to 0.00 ppm.



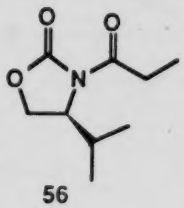
The broad peaks were attributed to rotational isomerism about the carbamate bonds of the CO_2CH_2Ph groups. In an attempt to improve the spectrum, variable temperature ^1H NMR studies were done. Heating of the sample to 374 °K in toluene-d_8 was required to cause the peaks to coalesce (figure 11). The complete spectrum could then be assigned using standard decoupling techniques. Such broad peaks were found to be a general property of the aminated carboximides; this makes the use of high temperature NMR a necessity for this class of compounds.



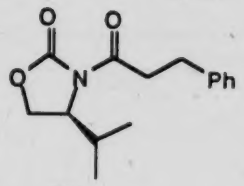
54



55



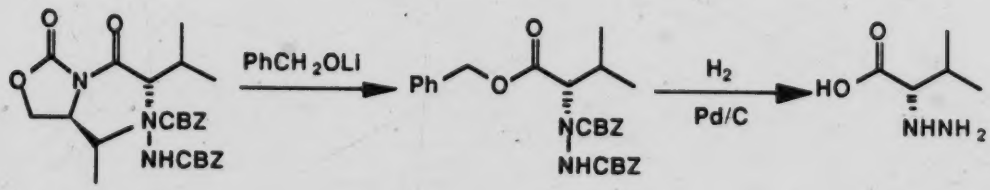
56



32

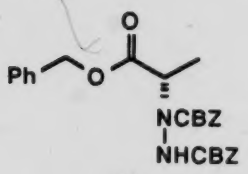
The analogs **54** and **55** corresponding to alanine and phenylalanine, respectively, were also prepared from the corresponding carboximides **56** and **32**. NMR and thin layer chromatography (TLC) appeared to show that compounds **53**, **54** and **55** were not contaminated with their undesired diastereomers. To test this, the aminated carboximides were cleaved with lithium benzylalkoxide[99] (cleavage of **53** is shown in scheme 19) at 0 °C in THF (10 min) to produce oxazolidinone **29** and the benzyl esters **57**, **58** and **59**, respectively. These esters were deprotected via hydrogenolysis with palladium catalyst to

Scheme 19.

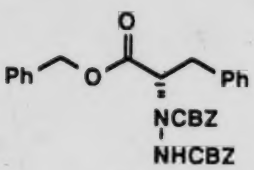


53 57 60

afford N-aminovaline (**60**), N-aminoalanine (**61**) and N-aminophenylalanine (**62**). Comparison of their optical rotations to literature values gave the enantiomeric ratios for the α-hydrazino carboxylic acids shown in table 2.



58



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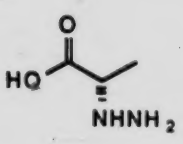
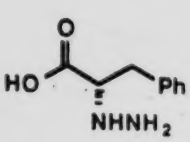
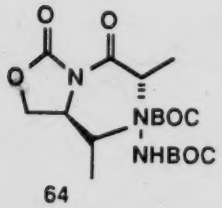


61

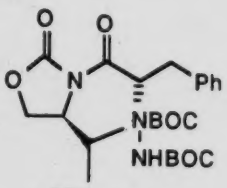


62

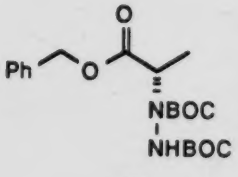
In order to improve the enantiomeric purity of the products, the steric bulk of the azodiformate was increased to enhance the diastereoselection observed during amination. Thus amination of the enolates of **56** and **32** with di-*tert*-butyl azodiformate (BOCN=NBOC, BOC = $COOC(CH_3)_3$) (**63**), (prepared by literature procedures[123,124]) gave the products **64** and **65** in good yield (table 2). These compounds were converted to



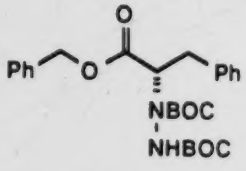
64



65



66

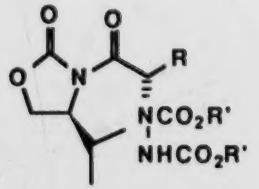


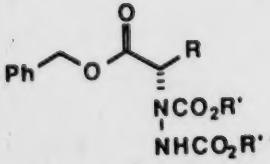
67

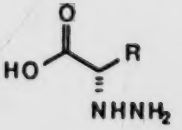
their benzyl esters, **66** and **67** respectively, as previously described and deprotected by consecutive treatment with trifluoroacetic acid and HCl/H_2O (scheme 20) to give alanine and phenylalanine analogs **61** and **62** respectively. The enantiomeric ratios for this pathway are also included in table 2.

Although enantiomeric purities were not as high as expected, some general trends became apparent. As the substituents at the β-position of the acyl side chain increased in size, higher enantiomeric purities were obtained. The size of the alkyl group on the azodiformate had a similar but smaller effect.

Table 2. Enantiomeric purities of α-hydrazinocarboxylic acids.



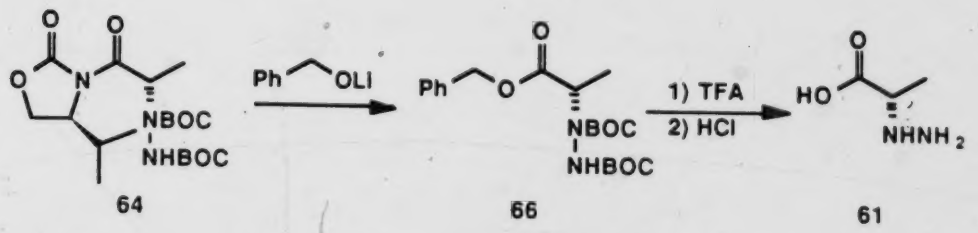




R	R'	Compound	Yield %	Compound	Yield %	Compound	Yield %	Enantiomeric Ratio
Me	CH_2Ph	**54**	91	**58**	93	**61**	98	72 : 28
Me	$C(CH_3)_3$	**64**	92	**66**	74	**61**	83	86 : 14
CH_2Ph	CH_2Ph	**55**	90	**59**	87	**62**	92	82 : 18
CH_2Ph	$C(CH_3)_3$	**65**	88	**67**	97	**62**	81	83 : 17
$CH(CH_3)_2$	CH_2Ph	**53**	85	**57**	86	**60**	98	97 : 3

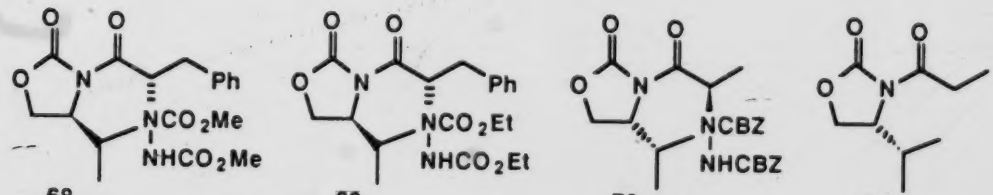
It was not clear if the low optical purities were due to a lack of diastereoselection in the initial amination or due to epimerization during lithium benzylalkoxide cleavage.

Scheme 20.



64 66 61

Standard chromatographic separations using either normal or reverse phase silica failed to resolve any of the aminated compounds into their diastereomeric constituents. However, HPLC analysis using a system suggested by David Evans (Chemistry Department, Harvard University, Cambridge, Massachusetts) successfully separated the diastereomers. Thus, the diastereoselectivity and the amount of epimerization during deprotection could be determined.

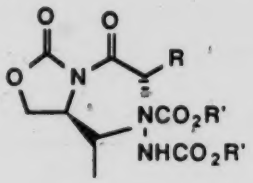


68 69 70 71

In order to further quantitate the effect of the size of the azodiformate alkyl side chain on diastereoselectivity, compounds **68**, **69** and **70** were prepared from their corresponding carboximides **32** and **71** as described previously. The data are displayed in table 3. From the HPLC results the trends in diastereoselectivity become clear. Increasing the steric bulk of the azodiformate improves the diastereomeric ratio (Me < Et < $CH_2Ph \approx$ *tert*-Bu) with little difference being observed between the benzyl and *tert*-butyl cases.

Similarily, greater size of the acyl side chain also increases the diastereoselectivity (Me < CH_2Ph < *iso*-Pr).

Table 3. Yields of amination and diastereomeric ratios of aminated carboximides.



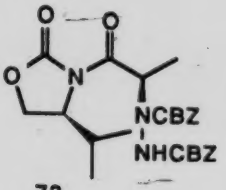
R	R'	Compound	% Yield	Diastereomeric Ratio
Me	CH_2Ph	**54**	91	90 : 10
Me	CH_2Ph	**70**[a]	79	90 : 10
CH_2Ph	Me	**68**	83	69 : 31
CH_2Ph	Et	**69**	88	75 : 25
CH_2Ph	CH_2Ph	**55**	90	94 : 6
CH_2Ph	$C(CH_3)_3$	**65**	88	93 : 7
$CH(CH_3)_2$	CH_2Ph	**53**	85	97 : 3

[a] compound **70** is the (*R*,*R*)-enantiomer of **54**

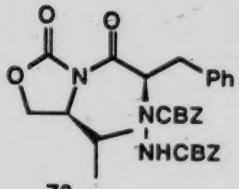
The reaction was extremely rapid with the dialkyl azodiformate being consumed as quickly as it was added. Cooling to temperatures as low as -110 °C did not alter the diastereomeric ratios significantly.

The unwanted diastereomers **72** and **73** of aminated carboximides **54** and **55** respectively, were isolated by medium pressure liquid chromatography (MPLC) or HPLC.

These compounds showed MS, ^{1}H NMR and IR properties that were quite different than those exhibited by **54** and **55**.



72

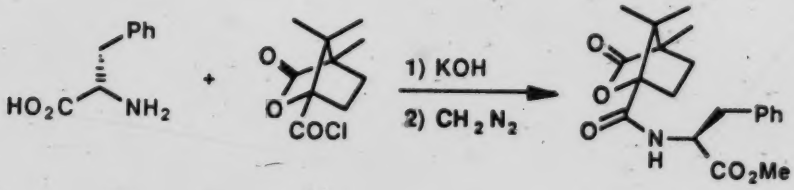


73

What is also obvious from tables 2 and 3 is that a fair amount of the wrong enantiomer in the final product results from cleavage with lithium benzylalkoxide. Because optical rotation is sensitive to small amounts of impurities that possess high optical rotations, another assay for enantiomeric purity was employed.

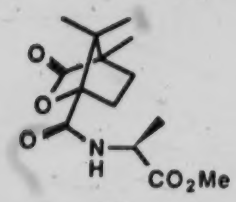
In principle, N-acylation of stereochemically impure α-amino or α-hydrazino acids with a chiral reagent would give two diastereomeric compounds which could be separated by gas chromatography. To test this idea, pure L-phenylalanine (**74**) was treated with commercially available (Aldrich) (1*S*, 4*R*)-(-)-camphanoyl chloride (**75**) according to the procedure of Armarego *et al*[125] and methylated with diazomethane to give the known[126] compound **76** (scheme 21). A mixture containing known amounts of L- and D,L-

Scheme 21.



74 **75** **76**

phenylalanine was also converted to the corresponding camphanamide methyl esters **77**. Gas chromatographic (GC) analysis of the mixture **77** produced two well separated peaks whose areas corresponded to the relative amounts of D- and L-isomers in the original amino acid mixture. The identity of the peaks was confirmed by coinjection with **76** and



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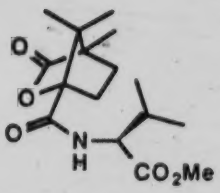
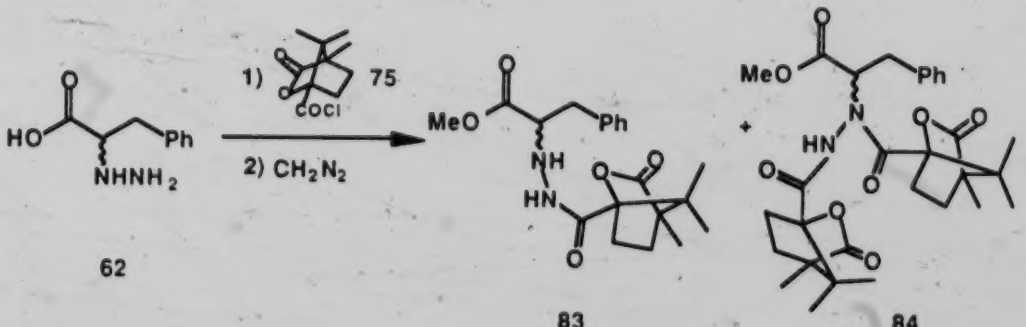


80

by mass spectrometry. The results indicate that this procedure is an effective method for stereochemical analysis of amino acids. In addition, the two enantiomers react equally well with the acid chloride and diazomethane (i.e. there is little or no kinetic resolution of enantiomers). Similarily, L-alanine (**78**) and L-valine (**27**) were converted to their camphanamide methyl esters **79** and **80**, respectively. The corresponding D,L mixtures **81** and **82**, respectively, were prepared. In each case gas chromatographic analysis was successful.

The approach was extended to the hydrazino acids. Addition of **62** to (-)-camphanoyl chloride (**75**) followed by diazomethane treatment not only resulted in the formation of the mono-camphanoylated product **83** but also bis-camphanoylated

Scheme 22.



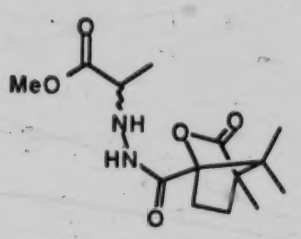
62

83

84

methyl ester **84** (scheme 22). However, formation of bis-camphanoylated compounds such as **84** could be eliminated by adding the (-)-camphanoyl chloride to a solution of the α-hydrazinocarboxylic acid. Compounds **85** and **86** were prepared similarly from **61** and **60**. The two diastereomers of **83** as well as those of **85** and **86** could be readily

separated by GC. The diastereomeric ratios of **83** and **86** determined by GC are in good agreement with the enantiomeric ratios of **62** and **60** obtained by optical rotations.



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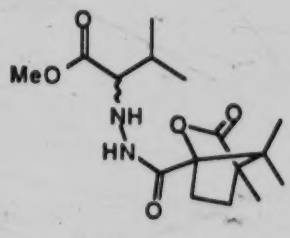
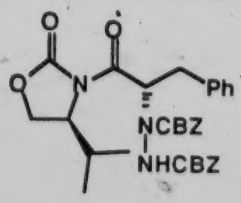


86

With a reliable assay for enantiomeric purity of the product α-hydrazinocarboxylic acids available, an epimerization study was undertaken. Thus, cleavage of the aminated carboximide **55** with lithium benzylalkoxide at room temperature for three hours afforded ester **59**. Hydrogenolysis to **62** and derivatization with (-)-camphanoyl chloride and diazomethane gave amide **83** which upon GC diastereomer analysis produced a ratio of 57 : 43 for the L and D isomers, respectively. Since **55** originally had a diastereomeric ratio



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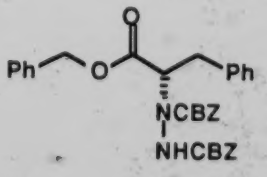
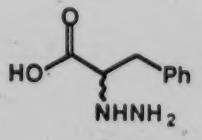
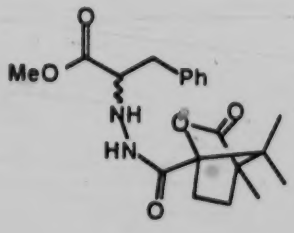


59



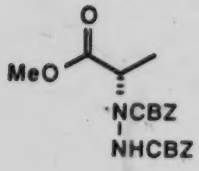
62



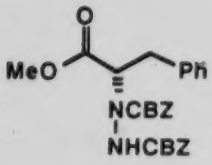
83

of 94 : 6 (by HPLC) it was concluded that epimerization at the α-position was indeed occurring during lithium benzylalkoxide cleavage. An earlier cleavage of **55** using the same reagents at 0 °C gave an 82 : 12 ratio (by optical rotation) (table 2).

In order to reduce the amount of epimerization, other nucleophiles were tested. Methanolysis of **54** and **55** with chloromagnesium methoxide according to the procedure of Evans *et al*[97] gave the methyl esters **87** and **88**, respectively. In both cases the



87

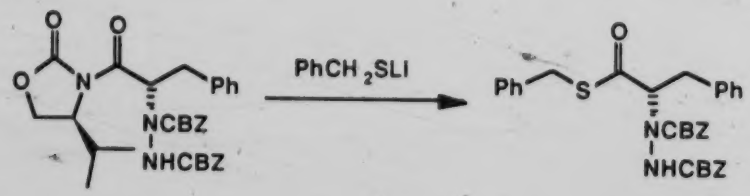


88

compounds had little or no optical rotation. It was assumed that these materials were nearly racemic.

Sulfur nucleophiles were investigated because of their increased nucleophilicity and decreased basicity.[127] Thus treatment of **55** with one equivalent of lithium benzylthiolate smoothly formed the thioester **89** (scheme 23) in 93% yield. Unfortunately the optical rotation of **89** was very low. Although benzylthiolate is less basic than the corresponding

Scheme 23.



55 89

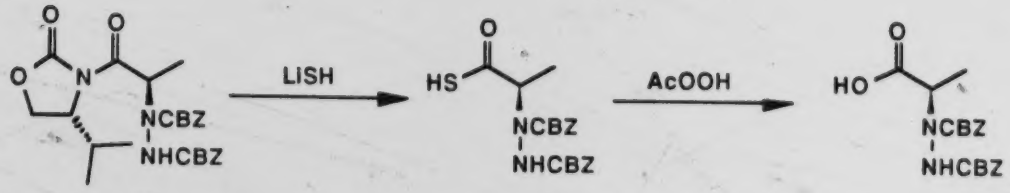
alkoxide, the resulting thioester is more prone to epimerization due to increased ketonic character of the carbonyl.[127] In addition, it was found that thioesters are difficult to hydrolyse without epimerization.[128] Since loss of stereochemistry with alkoxide and benzylthiolate probably occurs after cleavage of the oxazolidinone moiety,[98] conditions were sought which would generate a non-epimerizable anionic derivative of the α-hydrazino acid.

Cleavage with hydroxide proved to be difficult,[128] and gave many side reactions and low yields. Cleavage with hydrosulfide anion (HS^-) was investigated because it was known to be a good nucleophile[127]. Reaction of the aminated carboximides with anhydrous lithium hydrosulfide has the additional advantage of generating the anion of the

thiol acid, which is much less prone to deprotonation at the α-position than an ester. Treatment of the thiol acid with a solution of peracetic acid (40% solution in water) and THF affords the carboxylic acid in high yield[127].

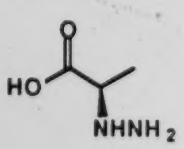
Thus, reaction of aminated carboximide **70** with an anhydrous suspension of lithium hydrosulfide afforded the lithium salt of thioacid **90** (scheme 24) which was

Scheme 24.



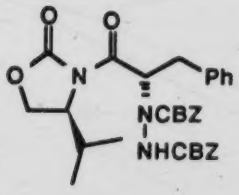
70 **90** **91**

converted directly, without prior purification, to the carboxylic acid **91** in 76% yield by reaction with peracetic acid. Catalytic hydrogenolysis of **91** gave D-N-amino alanine (**92**) that had an enantiomeric ratio (by GC analysis of its camphanamide methyl ester) that agreed with the diastereomeric ratio (HPLC) of the original carboximide **70**.

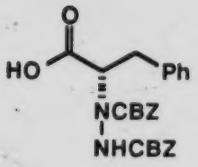


92

Similarily, **55** was converted to its corresponding carboxylic acid **93** in 85% yield. Hydrogenolysis followed by formation of the camphanamide methyl ester **83** and GC analysis as before gave an enatiomeric ratio of 87 : 13 for **62** that was identical within experimental error to the diastereomeric ratio of **55** from which it was derived. In the cases studied, lithium hydrosulfide cleavage offered an alternative method of deprotection that proceeded in high yield and with little or no racemization.



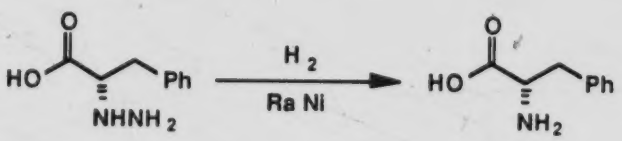
55



93

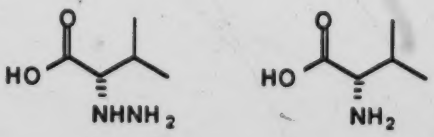
Previous work[129,130] had demonstrated that Raney nickel[131] catalyzes the reductive cleavage of the nitrogen-nitrogen bond of hydrazine derivatives. This approach was used to convert the α-hydrazinocarboxylic acids **20** to their parent α-amino acids **19**. Treatment of **62** with Raney nickel and hydrogen gas at 400 psi for ten hours gave L-phenylalanine (**74**) in quantitative yield (scheme 25). Conversion of **74** to its camphanamide methyl ester

Scheme 25.



62 74

gave a material that was chromatographically (GC) identical to the authentic standard **76** and showed that no detectable racemization had occurred during reduction. Similar results were obtained for N-aminovaline (**60**) which produced, after hydrogenolysis, L-valine (**27**) with no racemization.



60 27

Hence the amination of enolates derived from chiral carboximides with dibenzyl or di-*tert*-butyl azodiformates provides an efficient route for conversion of carboxylic acid derivatives to both α-hydrazinocarboxylic acids and α-amino acids of high stereochemical purity.[132]

Chapter 3

Biosynthetic Studies Using Stable Isotope NMR Techniques

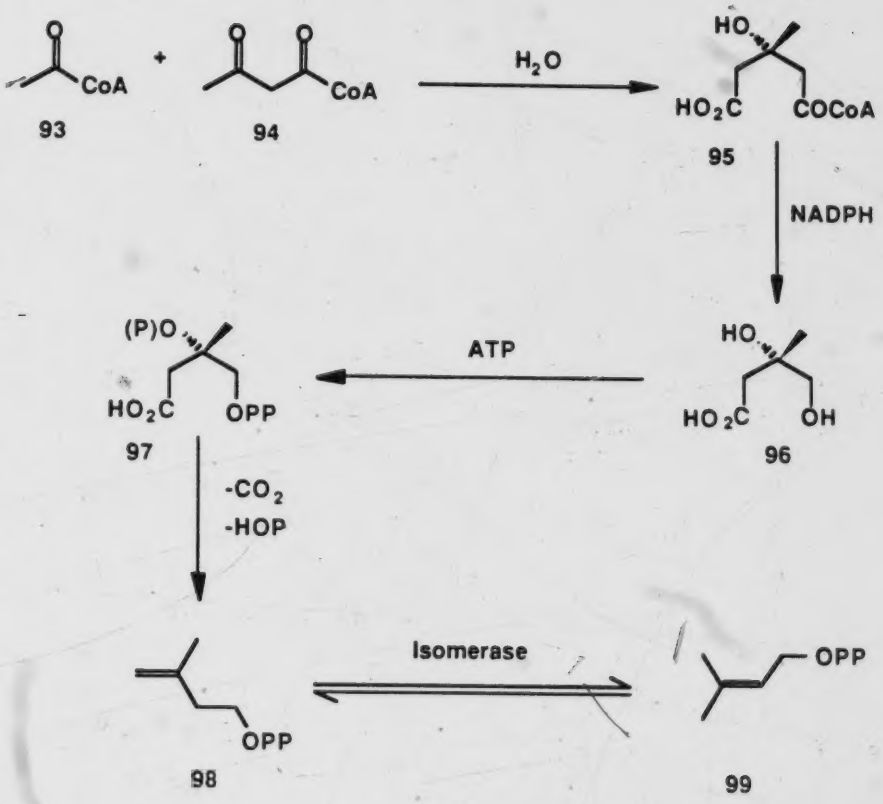
I. Introduction

The chemistry of natural products has been extensively studied for over one hundred years. However, the origins of natural product biosynthetic studies are much more recent.[133,134] It is customary to divide the study of metabolism into two fields, primary and secondary, although the dividing line is often obscure. The former concerns itself with the utilization and synthesis of essential chemical species: sugars, amino acids, fatty acids, nucleotides and their derived polymers. In contrast, secondary metabolism provides routes to compounds that often have no apparent utility and are found to occur in only a limited number of organisms. Secondary metabolism is divided into four main pathways based on the starting materials from which the metabolite is derived. These include the polyketide pathway, the shikimic acid pathway, amino acid derived metaboliism and the mevalonic acid pathway.

The mevalonic pathway produces a class of compounds (the terpenes) that formally appear to arise from isoprene. The isoprene rule, first postulated by Ruzicka, states that terpenes arise from the head to tail linkage of isoprene units to give metabolites that contain multiples of 5 carbon atoms.[134] Deviation from this number is the result of later modification. The biological source for the C_5 unit was identified as 3(*R*)-mevalonic acid (**96**) (scheme 26). The origin of **96** is acetate-based and arises from the condensation of acetyl CoA (**93**) with acetoacetyl CoA (**94**) in a branched fashion to give **95**, which is then reduced by NADPH. Decarboxylative elimination of phosphate from diphosphorylated mevalonic acid **97** gives isopentenyl pyrophosphate (**98**). Compound **98** is then reversibly isomerized to dimethylallyl pyrophosphate (**99**), the starter unit of terpene biosynthesis. Head to tail coupling of **98** and **99** begins formation of the carbon skeleton of mevalonic acid derived metabolites.

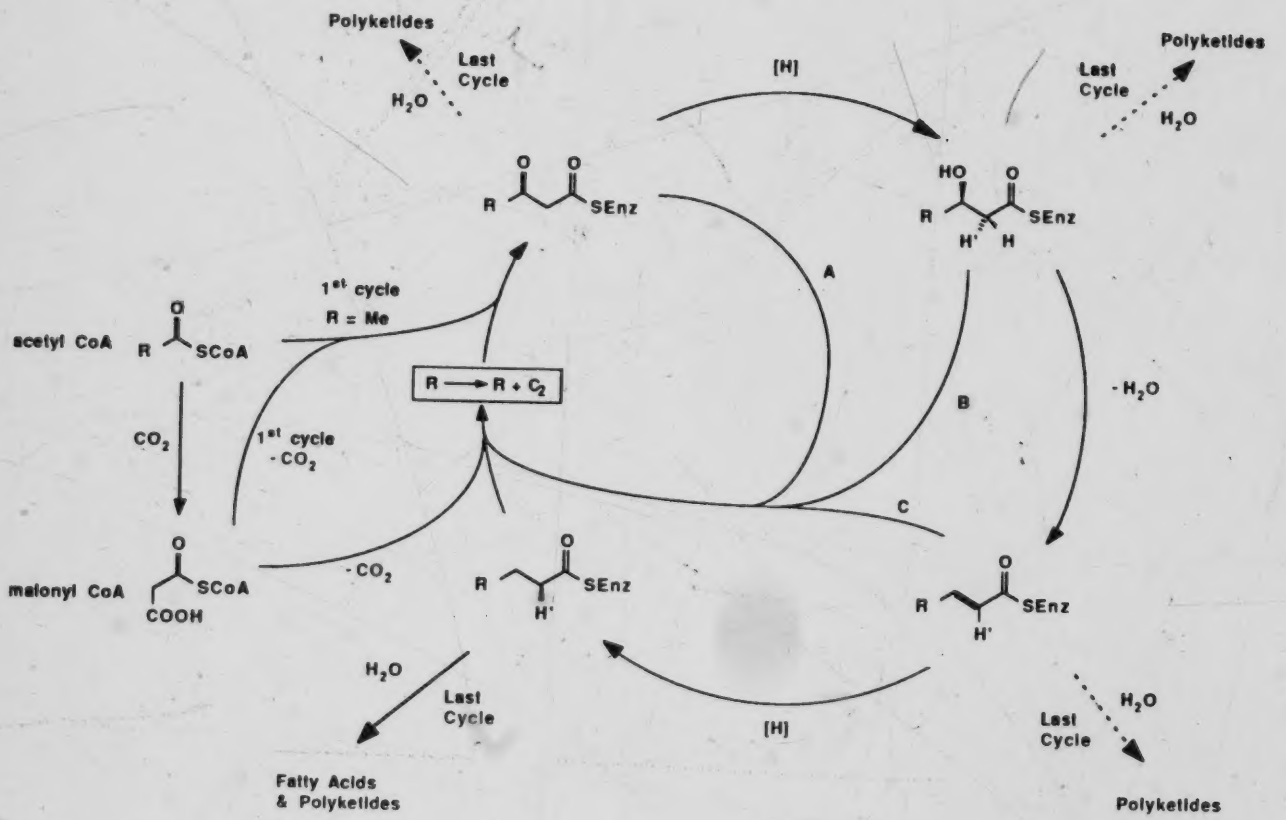
Polyketides and fatty acids arise from acetyl CoA metabolism by related mechanisms. Fatty acids are built up by the condensation of two-carbon units derived from

Scheme 26.



acetyl CoA on the multisubunit enzyme fatty acid synthetase[135-139] (figure 12). Biosynthesis of fatty acids begins by condensation of acetyl CoA and malonyl CoA with concurrent loss of CO_2 to give a β-ketobutyryl thioester. NADPH reduction of the β-keto group in stereoselective fashion affords the hydroxy thioester. Syn elimination of water to give the α,β-unsaturated system followed by a second reduction with NADPH produces the saturated enzyme bound thioester. The reduction is stereospecific with the position of the new hydrogen at C-2 being organism dependent. The chain is then added to a second molecule of enzyme bound malonyl CoA, increasing its length by two carbons. Repetition of the cycle adds a CH_2CH_2 unit with each circuit. Thus, acetate units are joined together in a head to tail fashion. Once seven or eight acetate units derived from malonyl CoA are

Figure 12. Fatty acid and polyketide biosynthesis[140].



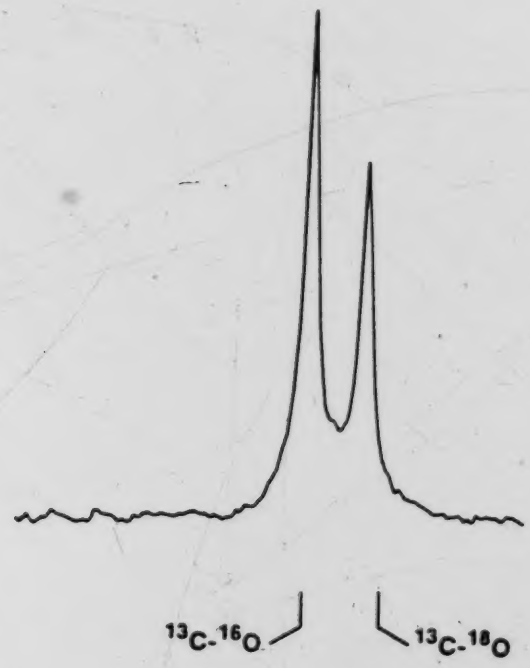
connected to the starter acetate unit, cleavage and/or hydrolysis occurs to give the C_{16} or C_{18} saturated fatty acid.

The Birch-Collie hypothesis postulates that the head to tail coupling of acetate units in polyketides is the result of a series of condensation-reduction steps analogus to fatty acid formation.[133,134] Failure to completely reduce the β-position of the enzyme bound thioester before the addition of the next acetate unit provides a mechanism by which carbonyl, hydroxyl, and olefinic groups (paths A, B, and C, respectively, in figure 12) may be introduced into the growing chain. The highly functionalized linear polyketide may then cyclize either while still bound to the enzyme or shortly after its release by hydrolysis. A consequence of this hypothesis is that the carbon-oxygen bonds of acetate would be expected to remain intact in the final metabolite. Although there is considerable evidence to support this "incomplete reduction" pathway,[140] an alternative mechanism must be considered. It is also possible that a partially or completely reduced fatty acid could have all of the functionality required to form the natural product introduced via separate oxidative enzymatic steps. Oxygen labelling studies can distinguish whether an organism synthesizes a metabolite via the reductive or oxidative paths, or via a combination of the two.

Two isotopes of oxygen are suitable for labelling studies: oxygen-17 and oxygen-18. The former has a nuclear spin (5/2), allowing it to be observed directly by NMR. However, its application to biological systems[141-144] has been limited by its expense due to low natural abundance (0.037%) and by broadness of NMR signals due to its quadrupole moment.

Unlike oxygen-17, oxygen-18 has no nuclear spin and cannot be directly observed by NMR. Early investigations[145-148] confirmed theoretical prediction[149] that isotopic substitution of an oxygen-16 with oxygen-18 induces an upfield shift in the ^{13}C NMR of the signal for the directly attached carbon atom.[150] For example, expansion of a peak

Figure 13. A typical ^{18}O induced isotope shift.



corresponding to a carbon 40% labelled with ^{18}O shows two signals (figure 13) in the high resolution ^{13}C NMR spectrum. The downfield peak is due to unlabelled material while the peak at higher field arises from the carbon bearing the isotopically substituted oxygen. Since the isotope shifts are small, unlabelled material acts as an internal standard.

The magnitude of the shift observed is dependent upon the hybridization and the electron density at the oxygen atom.[150] Thus, carbonyls tend to show larger shifts (55 - 30 ppb) (ketones > aldehydes > esters > amides) than do alcohols and ethers (35 - 10 ppb) (tertiary > secondary > primary > phenols). The shifts have been shown to be additive upon multiple ^{18}O substitution. The magnitude of the β-isotope shift induced by oxygen-18 on carbons two bonds away is generally much smaller (~5 ppb) but in unusual cases can be as great as 25 ppb.[151] Substitution with oxygen-18 does not measurably affect the relaxation times of the directly bonded carbon. Thus, integration of the peak areas gives

the relative amount of isotopic substitution. Since this technique determines the position of ^{18}O labelling, it has found application in mechanistic[152-155] and biosynthetic studies.[150]

Precursors doubly labelled with ^{13}C and ^{18}O allow "bond labelling" studies to be performed. Observation of an isotope induced shift is indicative of a carbon-oxygen bond in the natural product that has remained intact during its biosynthesis from the labelled precursor.[150] Because of its utility, this technique has been used extensively in biosynthetic studies.[156-161] Such double labelling not only allows the source of oxygens in a metabolite to be determined, but also provides information about the enzymatic mechanisms of formation of a natural product.

Labelling studies with isotopes of hydrogen can also give insight into chemical and biological mechanisms.[162,163] The most commonly used isotope, deuterium, is observed by changes induced in ^{1}H NMR spectra, directly by ^{2}H NMR,[164] or indirectly by isotope induced shifts in the ^{13}C NMR of carbons directly attached to deuterium (α-isotope shifts)[143,162,165-167] or two bonds away (β-isotope shifts).[140,165,167-169]

If deuterium enrichment is high, reduction of peak areas in the ^{1}H NMR can identify the site of labelling. However, enrichments are frequently low in biosynthetic experiments and differences due to labelling are difficult to observe. Deuterium NMR is often used to detect isotopic substitution. However the information available is often limited by extensive overlap of signals due to the quadrupolar nature of this atom. This problem can occasionally be overcome by the addition of lanthanide shift reagents.[170-172]

Observation of directly bonded deuterium in the ^{13}C NMR spectrum via an α-isotope shift often proves to be difficult. The low levels of incorporation usually obtained in biosynthetic studies give rise to weak α-isotope shifted signals. The intensity of the shifted peaks is further reduced by carbon-deuterium coupling, quadrupole broadening, and slow relaxation. In addition to this, if the carbon is fully deuterated the lack of nuclear Overhauser enhancement (NOE) associated with broad band ^{1}H decoupling further decreases the peak intensities. To circumvent this problem, Staunton *et al* [168] employed

sodium [1-^{13}C,2-$^{2}H_3$]acetate incorporation in conjuction with ^{13}C NMR spectroscopy. This precursor produces metabolites that display β-isotope shifts free of deuterium coupling and relaxation problems. Also, since the β-carbon bears no deuterium it can exhibit the full NOE expected from broad band ^{1}H decoupling. Unfortunately the magnitude and sign of the β-shift is unpredictable. At any given carbon it may be positive, negative or zero.[165] Hence, extra precautions must be taken to avoid misassignment of deuterium position and content.

Determination of which of two diastereotopic hydrogens on a methylene group is labelled with deuterium offers additional insight into the mechanisms by which secondary metabolites are assembled. Unfortunately the above techniques do not lend themselves well to this problem. Low incorporation rates and quadrupole line broadening often eliminate ^{1}H and ^{2}H NMR as usable techniques. The use of normal ^{13}C NMR for stereochemical analysis is even more limited since deuterium induced isotope shifts are often similar for each of the two diastereotopic methylene hydrogens. The excellent spectral characteristics of tritium NMR[173] would make this isotope ideal for such studies if the accompanying high levels of radioactivity did not restrict its use. However, a few biosynthetic studies employing this nucleus have been carried out.[16,173-175]

The advent of modern pulsed NMR experiments[176,177] has brought about new means of defining the position and extent of deuterium labelling. Several techniques[178-181] have been applied to deuterated systems including carbon-deuterium two dimensional shift correlation[182] and correlation of deuterium with protons.[183,184] The recent introduction of instrumentation allowing simultaneous pulsing of hydrogen, deuterium and carbon-13 while locking on fluorine, combined with modern two dimensional pulse methods, now permits determination of diastereotopic hydrogen labelling on methylene groups.[185,186]

II. Results and Discussion

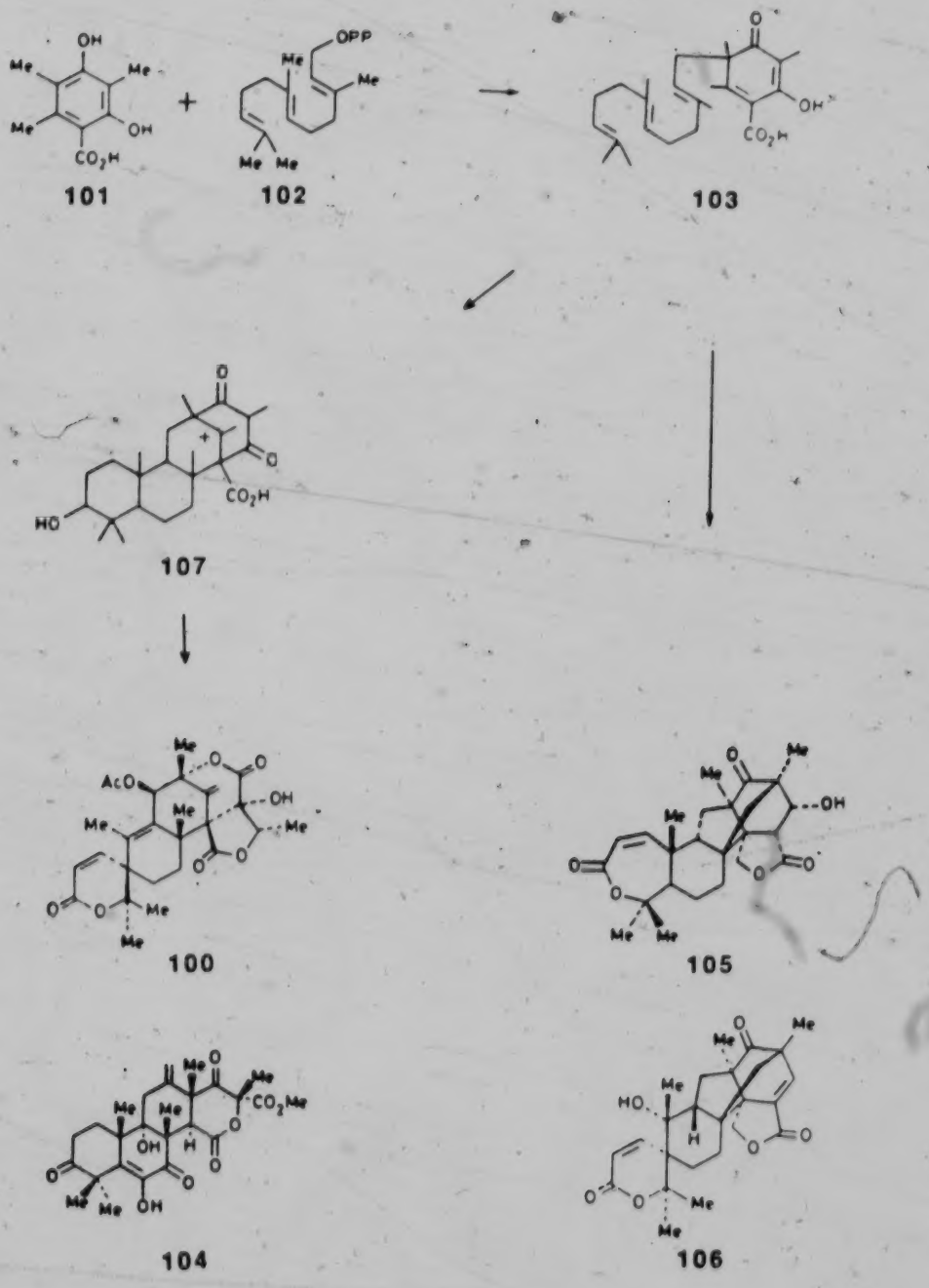
One-Dimensional NMR Techniques

In the following discussions, all fermentations and isolations were done by the group of Dr. Thomas J. Simpson. Austin (**100**), a toxic metabolite of *Aspergillus ustus*, has been shown to arise via a mixed terpenoid/polyketide (meroterpenoid) biosynthetic pathway[187,188] (figure 14). The key step in its biosynthesis involves the alkylation of the tetraketide 3,5-dimethylorsellinic acid (**101**) with farnesyl pyrophosphate (**102**) to give intermediate **103** which is a common precursor not only to austin (**100**) but also to the biosynthetically related metabolites terretonin (**104**), andilesin A (**105**), and andibenin B (**106**). Incorporation of labelled **101** directly into austin (**100**) and terretonin (**104**) has demonstrated that it is a specific precursor.[189]

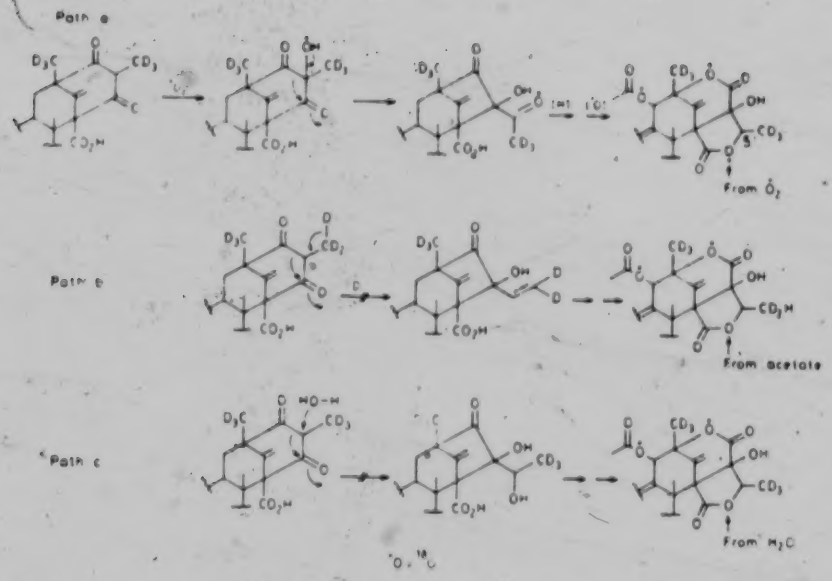
The conversion of **103** to austin (**100**) via **107** requires extensive oxidative modification. Several reasonable chemical pathways shown in scheme 27 can be differentiated since they require different origins for the oxygen and hydrogen atoms. For example, in path a aerobic oxidation followed by an α-ketol rearrangement affords the ring contracted intermediate. Reduction of the newly formed carbonyl to an alcohol and lactonization followed by a biological Baeyer-Villiger type reaction would give the upper ring portion of **100**. This pathway requires that oxygen at C-5' originates from the atmosphere. In path b the same oxygen is derived from acetate used to form the carbon backbone. In addition, this mechanism requires the loss of a hydrogen atom from the C-5' methyl group of **100**. Water from the medium is the source of the C-5' oxygen in path c.

To distinguish between the paths $^{18}O_2$ gas, sodium [1-^{13}C, $^{18}O_2$]acetate, and [Me-^{13}C,$^{2}H_3$]methionine were separately administered to *A. ustus* by our collaborator Thomas J. Simpson (University of Edinburgh) to give labelled austins.[190] Labelled acetate failed to enrich any of the oxygens in **100** with the exception of the acetyl carbonyl at position 11. However, $^{18}O_2$ gas was incorporated into the positions indicated in table 4.

Figure 14. Origin of the carbon skeleton of the meroterpenoids.



Scheme 27.



This oxygen labelli g pattern coupled with the retention of three deuteriums at the C-5' methyl group from methionine rules out paths b and c and is consistent with path a.

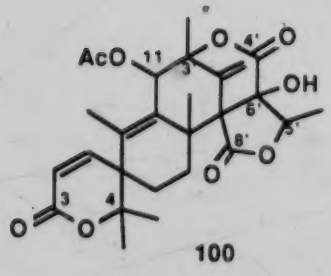
The introduction of the 11-acetoxy group probably occurs via allylic hydroxylation followed by acetylation. Incorporation of labelled acetate was too low at C-4', 6' and 8' for reliable observation of ^{18}O shifts. In order to determine the source of the oxygens at these sites, a 1 : 1 mixture of ethyl 3,5-dimethylorsellinate doubly labelled with ^{13}C and ^{18}O at either the 6 position or the carbonyl of the 7 position was synthesized by literature procedures.[191] This compound will be referred to as $^{13}C,^{18}O$-dimethylorsellinate (**108**) in all further discussions. Compound **108** was incorporated as before to give **100**. The labelling pattern observed was consistent with path a and clearly showed that the 6'-hydroxyl and C-8' carbonyls are derived from **108** (table 4).

As expected, both oxygens of the spiro-lactone A/B ring system are labelled by $^{18}O_2$ gas, thereby indicating that they are added in oxidative processes. The mechanism of

Table 4. ^{18}O isotopically shifted resonances of austin (**100**) enriched with $^{18}O_2$ gas and ethyl ^{13}C,^{18}O-dimethylorsellinate (**108**).

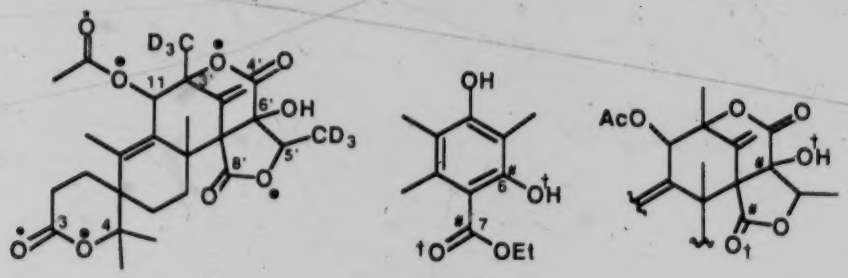
Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
8'	170.8	12 (55 : 45)
		38 (35 : 65)[a]
4'	170.2	13 (57 : 43)
$CH_3\underline{C}O$	168.4	14 (56 : 44)
3	163.6	10, 37, 47 (45 : 13 : 11 : 33)
4	85.6	43 (67 : 33)
3'	84.4	38 (60 : 40)
6'	80.8	8.3 (63 : 37)
		20 (37 : 63)[a]
5'	78.9	31 (66 : 34)
11	74.9	27 (67 : 33)

[a] enriched by **108**, all others enriched by $^{18}O_2$



100

formation of the lower rings of **100** remains speculative. A biosynthetic route consistent with the both carbon and oxygen labelling patterns is proposed in scheme 28. Oxidation of the A ring alcohol to a ketone followed by introduction of a $\Delta^{1,2}$ double bond would afford an α,β-unsaturated system. Baeyer-Villiger type oxidation and elimination would then

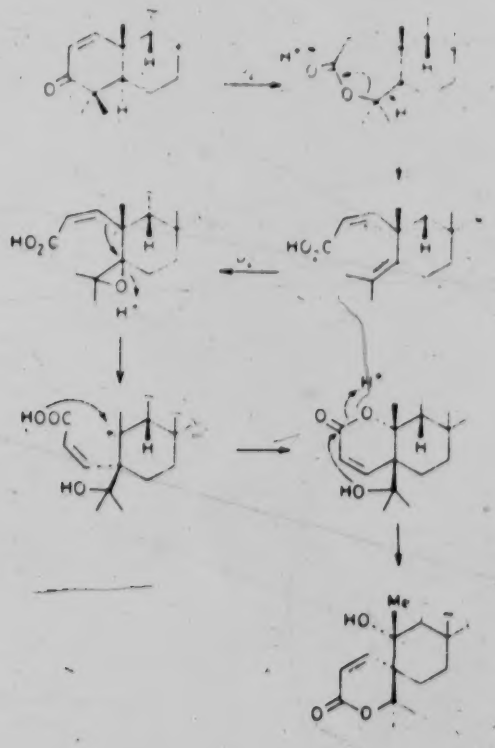


100 **108** **100**

• = acetate
• = oxygen gas

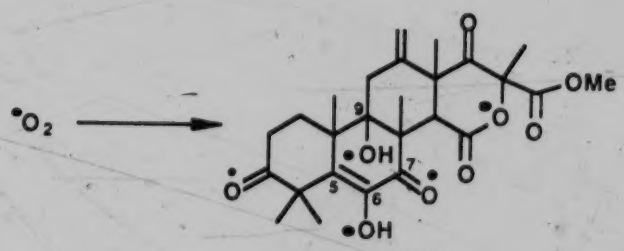
generate an alkene which can be epoxidized. Carbon-carbon bond migration would give a

Scheme 28.



carbocation that could cyclize by trapping the acid. Translactoniztion and elimination of water would produce **100**.

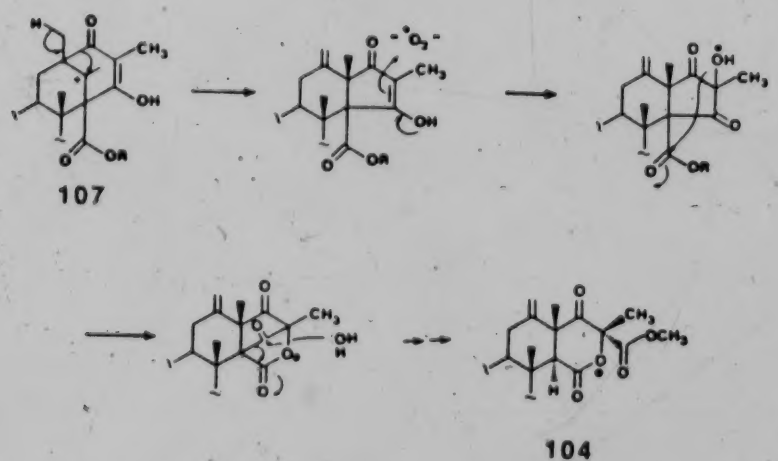
Terretonin (**104**), a metabolite from *Aspergillus terreus*,[192] has also been proposed to arise from **103** and **107** (figure 14) based on the carbon-13 acetate labelling pattern[193] and incorporation of ethyl dimethylorsellinate.[189] The proposed biosynthetic pathway for the formation of the C/D rings (scheme 29) involves an acyl shift with the loss of a proton



104

to generate the required exocyclic double bond. Aerobic hydroxylation, intramolecular lactoniztion and hydrolytic carbon-carbon bond cleavage would afford an intermediate

Scheme 29.



107

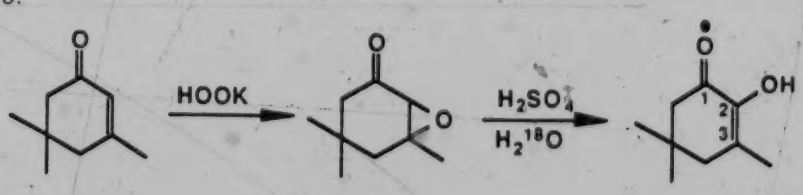
104

carboxylic acid which could be methylated by methionine to give **104**. Labelling studies with $^{18}O_2$ gas gave labelled terretonin **104** which displayed isotope shifts at the positions expected based on the proposed biosynthetic mechanism (table 5). However, a large apparent isotope shift (14 ppb) was observed at position 5 in the ^{13}C NMR spectrum.

The origin of this shift was unclear. It seemed possible that the C-9 hydroxyl group of **104** had added to the α,β-unsaturated ketone in a Michael type fashion, and that labelling from this oxygen was being observed. However, examination of the published x-ray structure[192] demonstrated that the hydroxyl in question points away from the unsaturated system, ruling out the possiblilty of bridging. Exchange of the enol proton with deuterium had no effect on the observed shift. Thus, the appearance of two signals in the ^{13}C NMR cannot be due to long range coupling of the β-carbon with the enolizable proton or to deuterium induced isotope shifts. The remaining possibility that this effect was a large ^{18}O-induced β-isotope shift was confirmed by studying model systems.

Compound **109** labelled with oxygen-18 in the carbonyl was prepared by acid catalyzed opening of epoxide **110** in the presence of $H_2{}^{18}O$. Compound **110** was obtained from cyclohexeneone **111** as described in the literature[194] (scheme 30). The labelled material **109** showed the expected shift at the carbonyl but no detectable isotope shift at either positions 2 or 3. Exchange of the enolic oxygen could not be carried out even under prolonged reflux in $H_2SO_4/H_2{}^{18}O$.

Scheme 30.



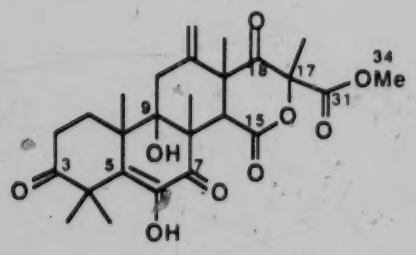
111 **110** **109**

Material labelled at the 2 positon, **112**, was obtained by treatment of **111** with oxygen-18 labelled potassium peroxide (**113**)[195] followed by rearrangement with sulfuric acid in

Table 5. ^{18}O isotopically shifted resonances of terretonin (**104**) enriched with $^{18}O_2$ gas and ethyl ^{13}C,^{18}O-dimethylorsellinate (**108**).

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
3	214.0	51 (70 : 30)
7	197.1	44 (61 : 39)
31	168.7	36 (42 : 58)[a]
15	167.7	8 (54 : 56)
		39 (66 : 34)[a]
6	138.8	14 (56 : 44)
5	131.7	14 (53 : 47)
17	85.6	25 (57 : 43)
9	77.7	28 (69 : 31)

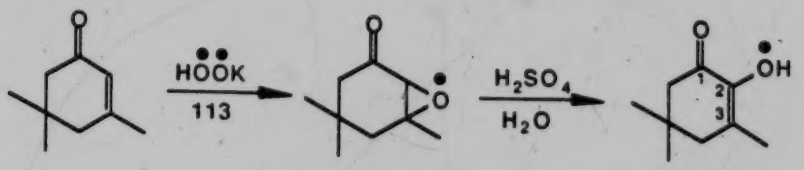
[a] enriched by **108**, all others enriched by $^{18}O_2$



104

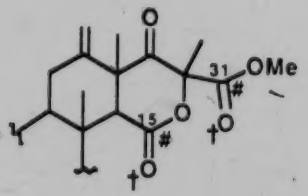
unlabelled water (scheme 31). In addition to the isotope shift observed at C-2 of 13 ppb a large β-shift of 11 ppb was found at C-3. This model study strongly supports the contention that **104** labelled by $^{18}O_2$ gas has an unusually large β-isotope shift at C-5.

Scheme 31



111 **112**

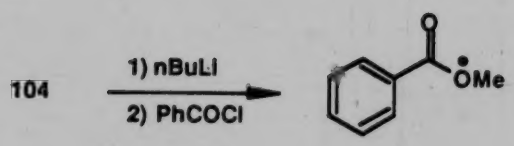
In order to determine the origin of the remaining oxygens, $^{13}C,^{18}O$-dimethylorsellinate **108** was fed to *A. terreus* to produce labelled terretonin **104** which displayed the presence of intact carbon-oxygen double bonds at positions 15 and 31 (refer to table 6). However, a smaller shift corresponding to labelling in the methoxy oxygen of



104

the ester linkage could not be resolved. To demonstrate labelling of this oxygen, **104** was treated with n-butyllithium and the liberated methoxide was trapped as its benzoate ester **114** by treatment with benzoyl chloride (scheme 32). GC/MS analysis of the semipurified reaction mixture indicated a 1.1% enrichment in the ^{18}O content of **114** which agreed well with the incorporation rate calculated from the NMR. Thus both oxygens at C-31 of terretonin (**104**) are equally labelled as expected for an intermediate free carboxylic acid. These results are in accord with the suggested biosynthetic mechanism (scheme 29).

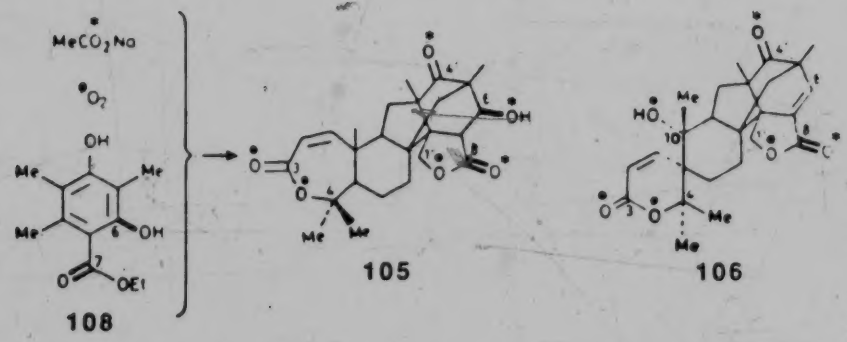
Scheme 32.



114

Andibenin B (**106**)[196-200] and andilesin A (**105**)[198,199,201,202], isolated from *Aspergillus variecolor*, were the final two meroterpenoids studied. The results of the incorporation experiments with $^{18}O_2$, sodium [1-^{13}C,$^{18}O_2$]acetate and ^{13}C,^{18}O-

Scheme 33.



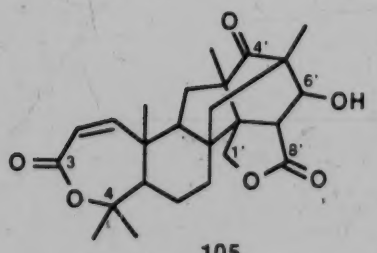
dimethylorsellinate **108** are shown in scheme 33 (tables 6 and 7).[203,204] A mechanism consistent with the observed isotope shifts and the carbon labelling pattern (scheme 34) involves oxidative cyclization of **103** (analogous to that of steroid biosynthesis) to give **115** containing an exocyclic double bond and the A/B ring system. A second cyclization could then occur to give **116**. Functionalization and rearrangement of the A/B rings (as previously described for austin (**100**)) and tautomerization followed by reduction of the D ring enol would then give andilesin A (**105**). Elimination of the elements of water from the newly formed D ring alcohol would afford andibenin B (**106**).

Although unrelated to the meroterpenoids, shamixanthone (**117**) and tajixanthone (**118**) are two metabolites of mixed polyketide/terpenoid origin also isolated from *Aspergillus variecolor*.[205-207] Previous incorporation studies with ^{13}C and ^{2}H suggest a

Table 6. ^{18}O isotopically shifted resonances of andilesin A (**105**) enriched with $^{18}O_2$ gas, sodium[1-^{13}C,$^{18}O_2$]acetate and ethyl ^{13}C,^{18}O-dimethylorsellinate (**108**).

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
4'	214.9	53 (84 : 16)[a]
8'	174.5	39 (82 : 18)[a]
		13 (90 : 10)[b]
		38 (88 : 12)[c]
3	166.2	47 (85 : 15)[b]
4	83.5	48 (87 : 13)[b]
6'	71.9	12 (83 : 17)[a]
		18 (72 : 28)[c]
1'	69.1	29 (88 : 12)[b]

[a] enriched by sodium [1-^{13}C,^{18}O]acetate
[b] enriched by $^{18}O_2$
[c] enriched by **108**

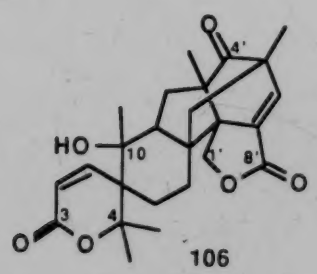


105

Table 7. ^{18}O isotopically shifted resonances of andibenin B (**106**) enriched with $^{18}O_2$ gas and ethyl $^{13}C,^{18}O$-dimethylorsellinate (**108**).

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
8'	169.0	15 (68 : 32)
		34 (87 : 13)[a]
3	165.4	50 (80 : 20)
4	86.9	42 (66 : 34)
10	79.3	32 (72 : 28)
1'	70.4	29 (70 : 30)

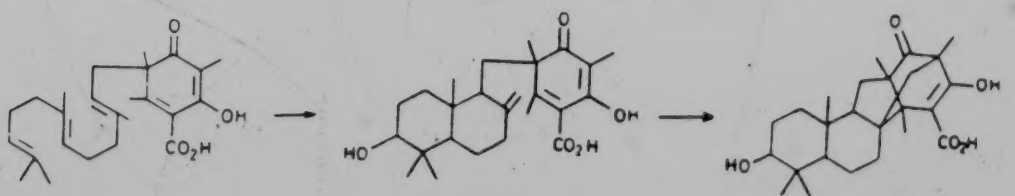
[a] enriched by **108**, all others enriched by $^{18}O_2$



106

biosynthetic pathway shown in scheme 35.[208-210] The carbon labelling pattern suggests that an octaketide precursor cyclizes to an anthrone which could then be hydroxylated, *O*-prenylated with dimethylallyl pyrophosphate (**99**), and oxidatively cleaved to give a benzophenone derivative. Observation of two distinct labelling patterns in the C ring suggests that the intermediate dihydroxyphenyl moiety is free to rotate. The xanthone ring could then form by Michael type addition/elimination of the A ring on the C ring (paths a_1 and b_1) or addition/elimination of the C ring on the A ring (paths a_2 and b_2). *C*-prenylation followed by aerobic oxidation to give the epoxide could then form tajixanthone (**118**). Since oxygen-18 induced iostope shifts have proven useful in determining the mode of xanthone ring formation in ravenelin[211] and sterigmatocystin,[212] $^{18}O_2$ gas was incorporated into tajixanthone(**118**) in collaboration with Dr. Thomas J. Simpson.[213]

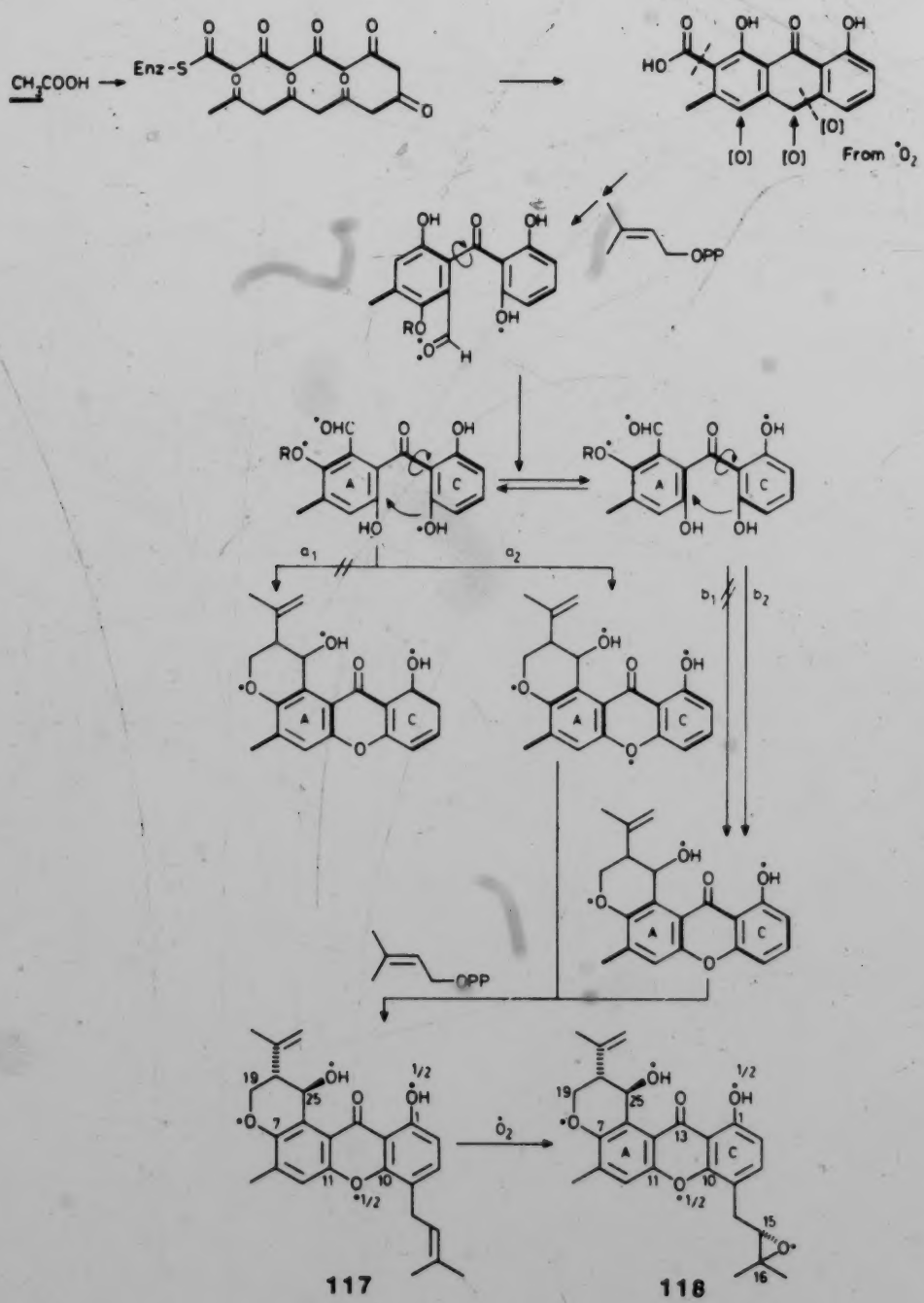
Scheme 34.



103 **115** **116**

Fermentation of *A. variecolor* under an $^{18}O_2$ atmosphere yielded tajixanthone which showed the presence of four ^{18}O atoms per molecule by mass spectroscopy. However, ^{13}C NMR analysis demonstrated the presence of ^{18}O at every oxygen with the exception of the C-13 carbonyl (table 8). Within experimental error, the relative amount of ^{18}O incorporated at C-1 and C-10 was half that of the other labelled sites. Combined with the mass spectral results, the data indicate that in any one molecule of **118** either the oxygen at C-1 or the one at C-10 (but not both) was labelled.

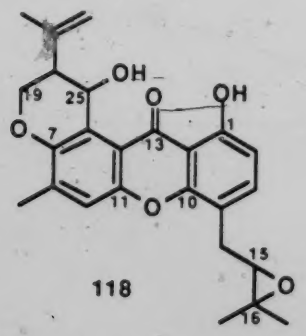
Scheme 35.



R = $CH_2CH{=}CMe_2$
OPP = Pyrophosphate

Table 8. ^{18}O isotopically shifted resonances of tajixanthone (**118**) enriched with $^{18}O_2$ gas.

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
1	160.4	10 (79 : 21)
10	152.9	23 (80 : 20)
11	152.0	24 (77 : 23)
7	149.5	15 (61 : 39)
19	64.5	23 (66 : 34)
15	63.2	33 (64 : 36)
25	63.1	15 (66 : 34)
16	58.5	41 (64 : 36)

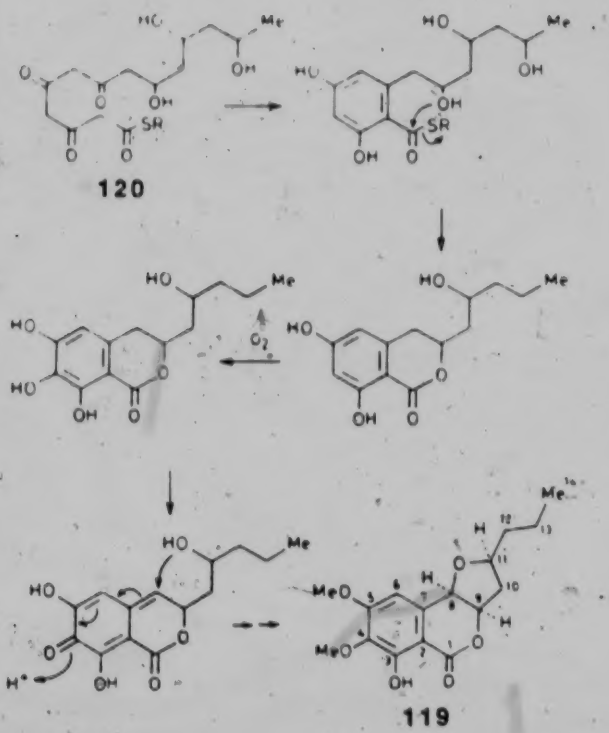


118

This is in agreement with the carbon labelling pattern[209] and is indicative of a freely rotating benzophenone that has an axis of symmetry as shown in scheme 17. Furthermore, cyclization must proceed by a Michael addition-elimination process of the C ring on the A ring (paths a_2 and b_2) since the reverse addition (paths a_1 and b_1) would give no label at the C-10 oxygen. In a complementary experiment [1-^{13}C,$^{18}O_2$]acetate was incorporated into **118** by *A. variecolor*. Unfortunately the incorporation rates were too low to observe any isotope shifts with the exception of the C-13 carbonyl.

Monocerin (**119**) is a metabolite having a polyketide origin that has been isolated from a variety of fungi.[214-217] Incorporation of carbon-13 labelled precursors suggested the biosynthetic pathway shown in scheme 36.[218] The uncyclized precursor **120** appears

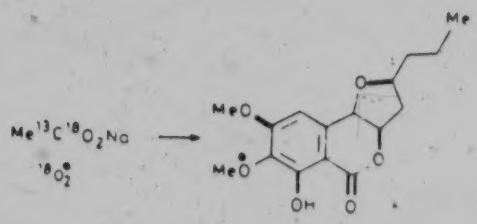
Scheme 36.



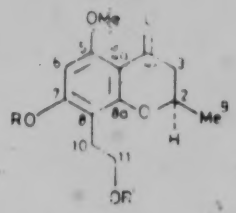
to be an ideal candidate for the "incomplete reduction" pathway that arises from the Birch-Collie hypothesis. To test this, $^{18}O_2$ gas and sodium [1-^{13}C,$^{18}O_2$]acetate were

incorporated into **119** by *Drechslera ravenelii* (in collaboration with T. J. Simpson). The labelling patterns from these incorporations are illustrated in scheme 37 and the ^{18}O isotope shifts are listed in table 9. As expected, all of the oxygens in **119** were derived from acetate with the exception of that at C-4 which arises via aerobic oxidation.

Scheme 37.



Incorporation of doubly labelled acetate into monocerin also offers an example of the "bond labelling" technique. For example, it is conceivable that cyclization of the B ring could proceed in one of two ways, either direct attack of the C-9 hydroxyl group on the thioester of the acyl-CoA complex (as depicted in scheme 18) or attack of the free carboxylate anion on C-9 bearing a good leaving group. The results of the labelling studies clearly show that the bond between C-9 and the oxygen has remained intact throughout the biosynthesis, thus eliminating the second alternative.



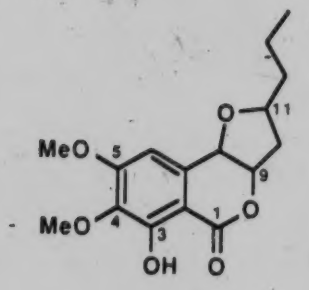
121 R = H
122 R = Ac

The fungal metabolite LL-D253α (**121**) isolated from *Phoma pigmentivora*[219] as its diacetate (**122**) contains an unusual hydroxyethyl substituent. Its structure suggests a polyketide origin. Labelling studies with sodium [1,2-$^{13}C_2$]acetate demonstrated that all of

Table 9. ^{18}O isotopically shifted resonances of monocerin (**119**) enriched with $^{18}O_2$ gas and sodium[1-^{13}C,$^{18}O_2$]acetate.

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
1	167.9	33 (64 : 36)
3	158.8	16 (63 : 37)
5	156.3	11 (59 : 41)
4	137.4	12 (61 : 39)[a]
9	81.4	31 (61 : 39)
11	78.7	30 (62 : 38)
4-OMe	60.7	26 (56 : 43)[a]

[a] enriched by $^{18}O_2$, all others enriched by sodium [1-^{13}C,$^{18}O_2$]acetate

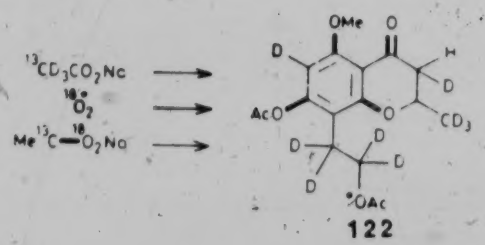


119

the carbons are derived from intact acetate units.[220] However, when sodium[1-^{13}C]acetate was incorporated, not only were C-2, C-4, C-5, C-7, C-8a and C-10 highly enriched as expected, but C-11 was also labelled. Analogously, C-10 was unexpectedly enriched by [2-^{13}C]acetate.

Incorporation of sodium [1-^{13}C,$^{18}O_2$]acetate into **121** and chemical conversion to diacetate **122** gave the labelling pattern shown in scheme 38 (table 10). Clearly the carbon oxygen bonds at C-4, C-5, C-7 and C-8a had remained intact from acetate during the biosynthesis. Oxygen-18 gas labelled the singly binded oxygen in the two carbon side chain, but the incorporation rate was significantly less than expected.

Scheme 38.



122

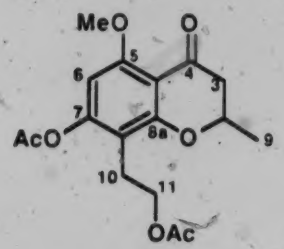
In order to further elucidate the mechanism by which this metabolite is formed, deuterium labelling was used. Sodium [2-^{13}C,$^{2}H_3$]acetate was incorportaed into **121** by *P. pigmentivora*. The normal carbon-13 broad band ^{1}H decoupled spectrum of the C-9 methyl group of the derived diacetate **122** is shown in figure 15a. This spectrum clearly illustrates the main difficulty in observing α-isotope shifts by carbon-13 NMR. The complex spectrum (a) is due to deuterium coupled signals from CH_3, CH_2D, CHD_2 and CD_3 methyls, and is exceedingly difficult to interpret.

To simplify the spectrum, broad band ^{2}H decoupling was simultaneously applied. Introduction of a small amount of hexafluorobenzene into the NMR tube and the use of a fluorine lock allowed sharp signals to be obtained. With decoupling, the spectrum simplifies to four equally spaced lines (figure 15b). These signals are easily assigned to CH_3, CH_2D, CHD_2 and CD_3 species, respectively, and illustrate the additivity of the α-

Table 10. Isotopically shifted resonances of LL-D253α diacetate (**122**) enriched with $^{18}O_2$ gas, sodium [1-^{13}C,$^{18}O_2$]acetate and sodium [2-^{13}C,$^{2}H_3$]acetate.

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
4	190.1	41 (92 : 8)[a]
8a	162.1	16 (80 : 20)[a]
5	159.9	17 (78 : 22)[a]
7	155.1	21 (84 : 16)[a]
6	98.9	298[b]
11	62.7	304, 610[b]
		27 (86 : 14)[c]
3	45.4	326, 369[b]
10	23.1	378, 557[b]
9	20.4	269, 540, 811[b]

[a] enriched by sodium [1-^{13}C,$^{18}O_2$]acetate
[b] enriched by sodium [2-^{13}C,$^{2}H_3$]acetate
[c] enriched by $^{18}O_2$ gas

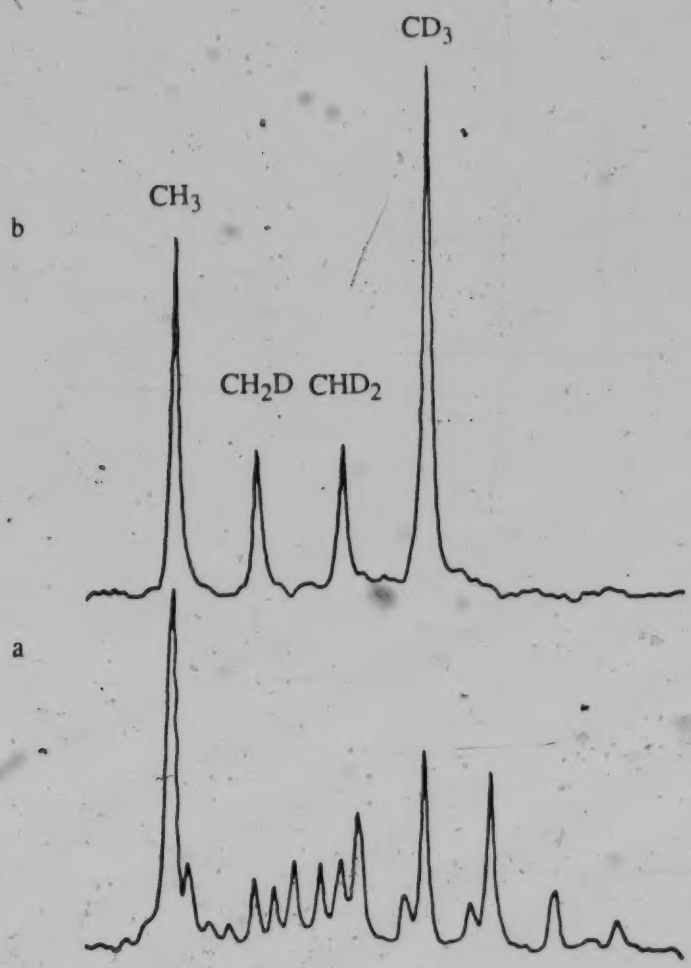


122

isotope shifts. The presence of the CD_3 group indicates its origin from an acetate starter unit.

Examination of C-3 showed the presence of two isotope shifts (326 ppb and 369 ppb) (table 10). The magnitude of the shifts are indicative of a carbon bearing one deuterium. However, the observation of two shifts of almost equal size can only be due to

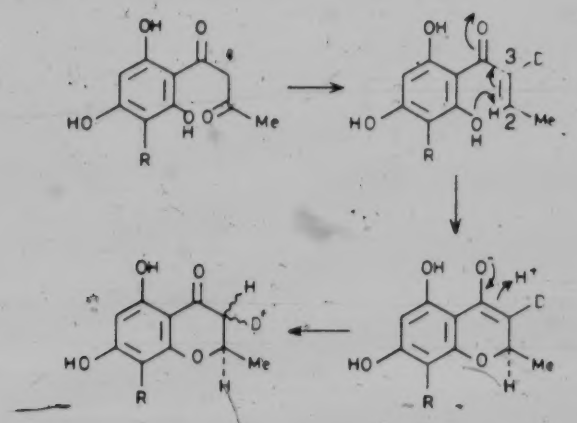
Figure 15. Normal and deuterium decoupled ^{13}C spectra of the C-9 methyl of deuterated LL-D253α diacetate **122**.



the presence of two species, each one being labelled in one or the other of the two diastereotopic methylene positions. Since the two shifted peaks have approximately equal area, the deuterium must be nonstereospecifically incorporated at this site.

These results indicate that the chromanone ring is formed by conjugate addition of a phenolic hydroxyl group to an α,β-unsaturated ketone (scheme 39). The 2*R* configuration of **121** indicates that the ring closure is stereospecific with respect to C-2. However, the presence of one deuterium at both diastereotopic C-3 positions demonstrates that

Scheme 39.

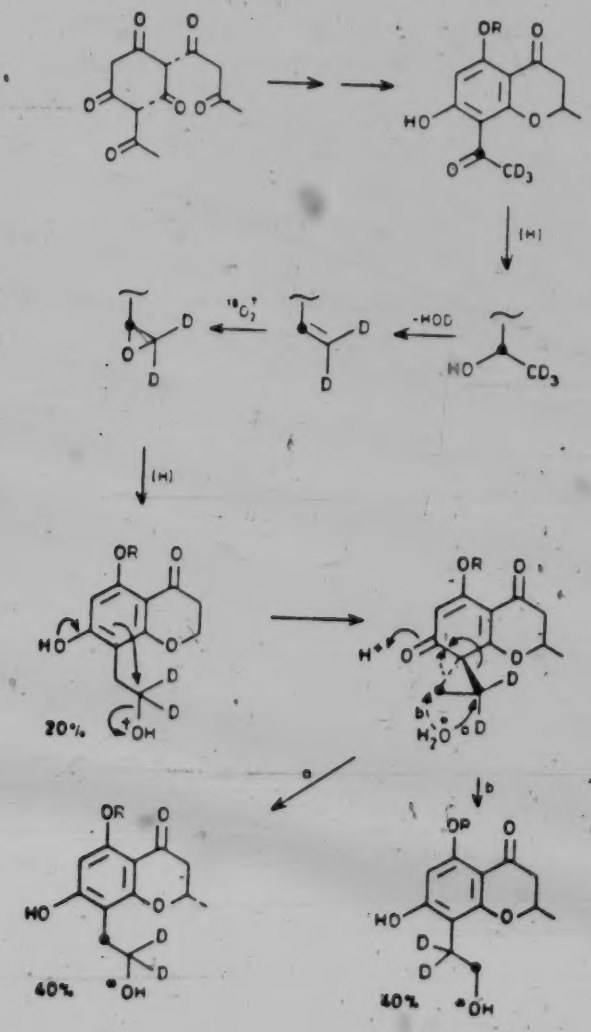


protonation of the intermediate enolate proceeds with equal facility from both sides of the molecule. This is in contrast with the chalcone to flavone ring closure which is known to be completely stereospecific.[221]

In addition to one deuterium at C-6, two deuteriums were incorporated at both C-10 and C-11. The ratio of deuterium at C-10 to C-11 was 40 : 60. Together with the carbon labelling pattern, this indicates some randomization of these carbons during the biosynthesis. To account for this, the biosynthetic pathway in scheme 40 was proposed. Two preformed polyketide chains of various possible lengths (i.e. C_8+C_4 as depicted in scheme 40, or C_{10}+C_2, or C_6+C_6) are joined prior to aromatization of ring a. The loss of acetate derived oxygen in the C_2 side chain, randomization of label, and introduction of

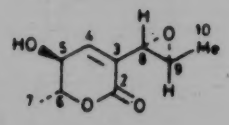
aerobic oxygen can be explained by a reduction and elimination process similar to fatty acid biosynthesis. The resulting olefin could then be epoxidized and reductively cleaved to give the hydroxyethyl group directly. To account for the 80% randomization, a spiro-fused cyclopropyl intermediate may form by attack of the phloroglucinol ring on C-11 with expulsion of the hydroxyl group. Hydrolytic opening of the cyclopropane at either site a or site b would produce the observed randomization of label. The remaining 20% of the natural product would be derived directly from the reduced epoxide. This is in accord with the low incorporation of $^{18}O_2$ at this site.

Scheme 40.



It is not clear whether the randomization of label is an *in vitro* or *in vivo* process. However, [10-$^2H_2$]-LL-D253α has been prepared and no randomization of label was observed upon mild acid or base treatment.

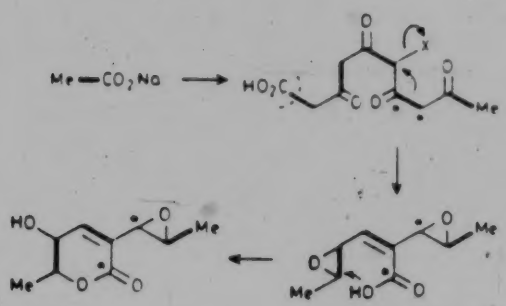
Aspyrone (**123**) is a metabolite from *Aspergillus melleus*[222] whose biosynthesis



123

has been extensively studied.[223,224] Incorporation of isotopically labelled acetates and malonates suggested decarboxylation and Favorski-type rearrangement of a linear pentaketide as shown in scheme 41. In support of this mechanism a two bond coupling between C-2 and C-8 is observed upon incorporation of sodium [1,2-$^{13}C_2$]acetate.[225]

Scheme 41.



123

In order to gain mechanistic insight into the formation of **123**, oxygen-18 labelling studies were carried out in collaboration with T. J. Simpson.[226] Incorporation by of sodium [1-^{13}C,$^{18}O_2$]acetate *A. melleus* into **123** gave high levels of ^{13}C enrichment. However, no isotope shifts due to ^{18}O were observed in the ^{13}C NMR spectrum. In the

complementary experiment, $^{18}O_2$ gas incorporation caused isotope shifts at all carbons bearing oxygen (table 11). Integration of the shifted peaks indicated that the epoxide and alcohol were both highly and equally enriched. C-2 showed two isotopically shifted signals corresponding to labelling in the carbonyl and in the ether oxygen of the lactone. Within experimental error, the intensities of these signals were half that observed for the epoxide and alcohol. A reasonable explanation of these observations is that one oxygen-18 atom has been aerobically introduced into a free carboxylic acid and that the remaining oxygen of the acid must have been derived from the medium. Cyclization of this acid would then give the lactone ring of **123**. A biosynthetic pathway in accord with the observed labelling pattern is proposed in scheme 42. Decarboxylation and epoxidation of a

Scheme 42.

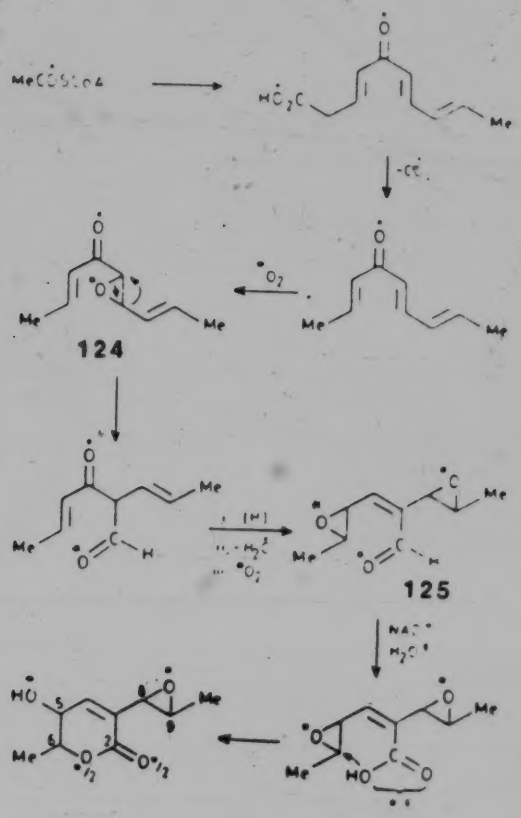
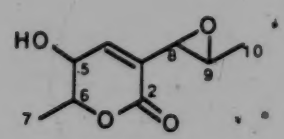


Table 11. Isotopically shifted resonances of aspyrone (**123**) enriched with $^{18}O_2$ gas and sodium [2-^{13}C,$^{2}H_3$]acetate.

Carbon	δ	Δδ/ppb (^{16}O : ^{18}O)
2	163.0	9, 37 (61 : 20 : 19)
6	79.3	31 (78 : 22)
5	67.6	16 (63 : 37)
		468[a]
9	59.1	34 (63 : 37)
8	54.6	31 (62 : 38)
7	18.0	262, 524[a]
10	17.6	270, 541, 812[a]

[a]enriched by sodium [2-^{13}C,$^{2}H_3$]acetate, all others enriched by $^{18}O_2$



123

pentaketide precursor obtained by a reduction/elimination pathway similar to fatty acid biosynthesis would give **124**. Rearrangement of the epoxide followed by reductive elimination of water and subsequent epoxidation would afford **125** which contains no label from acetate. The aldehyde could then be converted via NAD^+ mediated oxidation to a carboxylic acid that can cyclize on the epoxide to give aspyrone.

Further support for this mechanism is supplied by incorporation of sodium [2-^{13}C,$^{2}H_3$]acetate. The broad band deuterium and proton decoupled ^{13}C spectrum indicated the presence of two deuteriums at C-7 in accord with the proposed decarboxylation. The presence of three deuteriums at the C-10 methyl group shows that it must be part of the acetate starter unit.

Two-Dimensional NMR Techniques

Deuterium decoupling now allows simple determination of the number of deuteriums that are directly bonded to a carbon by observation of the associated α-isotope shifts in ^{1}H-decoupled ^{13}C NMR spectra. However, only in rare cases such as LL-D253α can any stereochemical information be obtained directly from such an experiment. Combination of this technique with modern two-dimensional pulse techniques, in particular ^{1}H,^{13}C shift correlation, offers a solution to this problem.

Camphor

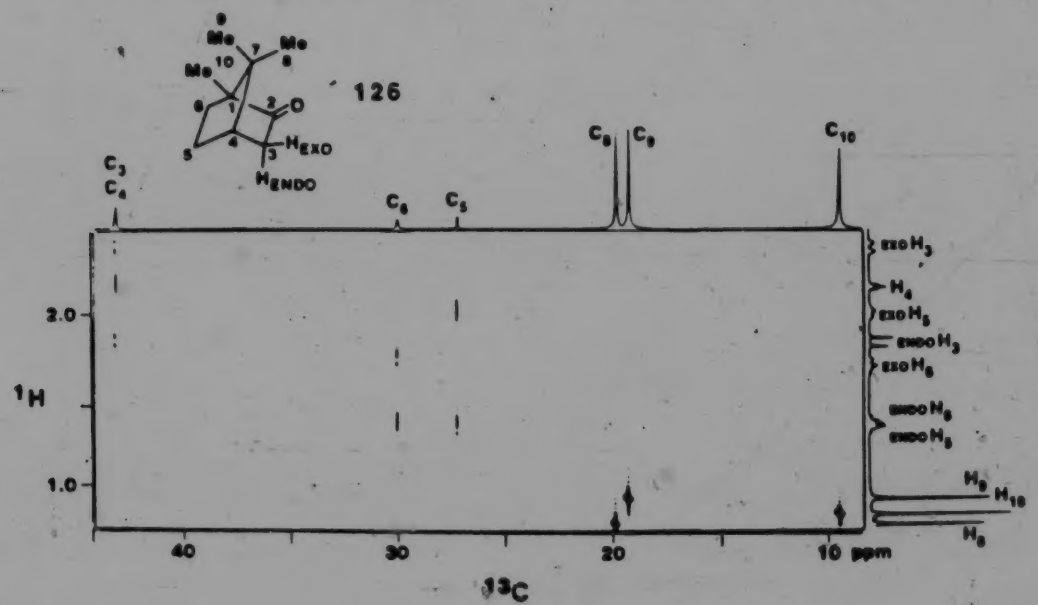
The first test case chosen was camphor (**126**) because the exchangeable C-3 methylene signals are well separated in the ^{1}H NMR spectrum (see table 12) but carbons C-3 and C-4 appear within 0.1 ppm of each other in the ^{13}C NMR spectrum. In addition, slight changes in temperature and concentration can shift their positions sufficiently to cause complete overlap. Because conflicting ^{13}C NMR assignments for **126** have been reported,[227-232] several techniques such as spin echo Fourier transform (SEFT)[233] and 2D-INADEQUATE[176,234,235] were used to correctly assign its ^{13}C NMR spectrum. A

Table 12. Chemical shifts of camphor (**126**)

Position	δ ^1H	δ ^{13}C
1	-	57.6 (s)
2	-	217.9 (s)
3	2.36 (exo)	43.4 (t)
	1.84 (endo)	
4	2.10	43.5 (d)
5	1.96 (exo)	27.4 (t)
	1.41 (endo)	
6	1.68 (exo)	30.2 (t)
	1.34 (endo)	
7	-	46.8 (s)
8	0.83	19.8 (q)
9	0.95	19.3 (q)
10	0.91	9.3 (q)

previous ^1H NMR assignment[232] was confirmed by heteronuclear proton-carbon shift correlation[176,236] (figure 16), homonuclear proton-proton decoupling, and Eu(fod)$_3$ induced shifts. The resulting assignments of chemical shifts are summarized in table 12 and agree fully with a recent NMR study of camphor (**126**).[237]

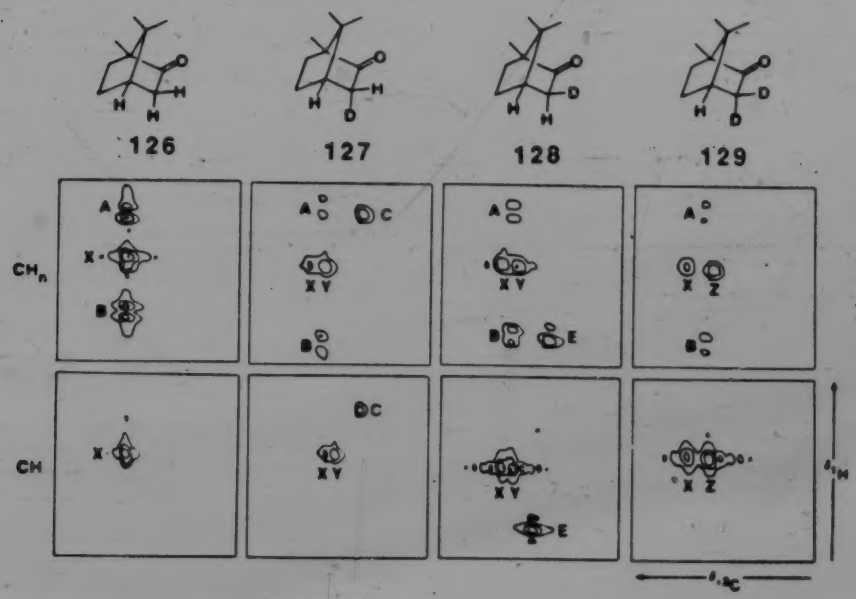
Figure 16. Normal ^1H,^{13}C shift correlation spectrum of unlabelled camphor (**126**).



Endo-, *exo-* and bis-deuterated camphors **127**, **128** and **129**, respectively, were prepared by Dr. Paul Reese using literature procedures[238,239] and were mixed in a 2 : 1 ratio (labelled : unlabelled) with **126**. The C-3 and C-4 portions of the deuterium decoupled two-dimensional $^{1}H,^{13}C$ chemical shift correlation for each of the camphors are shown in figure 17. The upper row of plots are the CH_n or full correlations obtained using standard conditions with simultaneous deuterium decoupling.[236] The lower row are the much simpler "CH only" correlations obtained by using a selective heteronuclear pulse sequence[240] with deuterium decoupling and the variable pulse angle θ set to 90°.

Examination of the full correlation plots shows three common signals (A, B and X). Signals A and B correspond to the correlation of the C-3 methylene carbon of

Figure 17. Deuterium-decoupled normal (CH_n) and selective (CH) proton/carbon shift correlation plots of the C-3 and C-4 region of camphor. In each plot the chemical shift increases from 1.76 to 2.43 ppm for ^{1}H on the vertical axis and from 42.76 to 43.93 ppm for ^{13}C on the horizontal axis (right to left).



unlabelled camphor **126** with its *exo* and *endo* hydrogens, respectively. Correlation of the C-4 methine with its proton gives rise to signal X. Substitution of the 3-*endo* proton of **126** with deuterium to give **127** caused two new signals, C and Y, to appear in both the CH_n and CH plots. Signal Y results from the C-4 methine being shifted upfield on the horizontal ^{13}C axis by the presence of a deuterium at C-3 (β-isotope shift).[165,168] Signal C arises from C-3 carbons bearing a single proton in the *exo* position being shifted upfield by the large α-isotope shift.[162,165] Similarily, the correlations of *exo*-deuterated camphor (**128**) give signals Y and E. Correlation of **129** gave only one new signal Z, corresponding to the C-4 methine being shifted upfield by the presence of two β-deuteriums.

Repetition of these experiments with mixtures of varying amounts of labelled compounds **127** and **128** and unlabelled camphor, allowed an estimate of the lower level of deuterium detection. Correlation of a sample containing 20% monodeuterated camphor and 80% unlabelled material **126** still gave clean correlation plots. These results combined with one-dimensional broad band $^{1}H,^{2}H$-decoupled ^{13}C NMR studies suggest that the lower limit of detection is in the range of 10% deuterium on natural abundance carbon-13.

N-Benzoylphenylalanine methyl ester

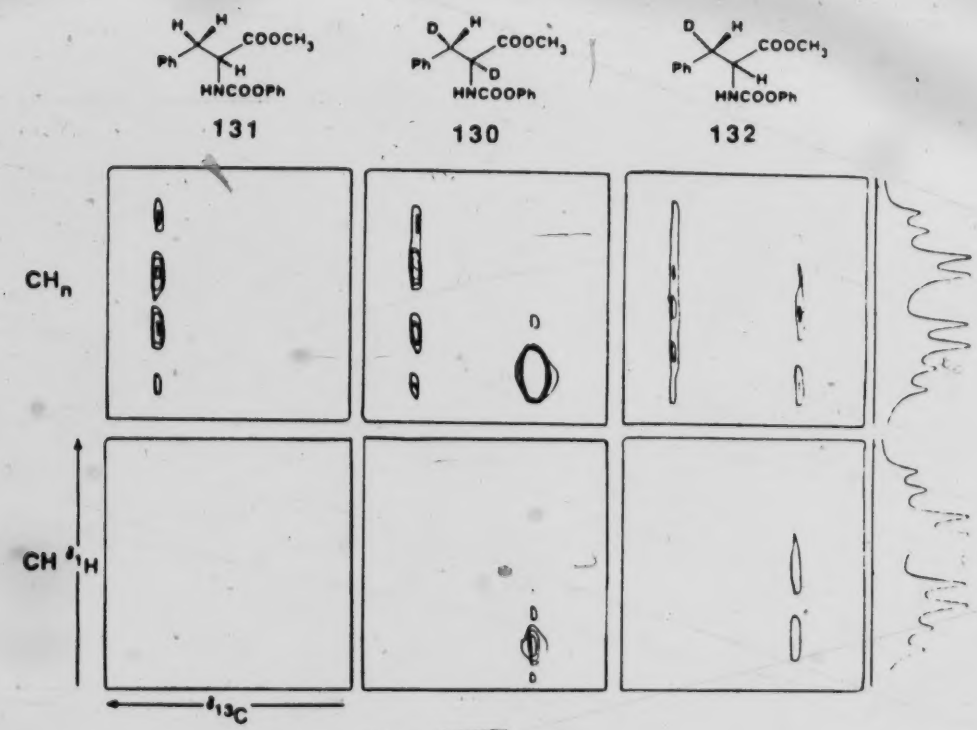
In most cases the methylene carbons are well separated in the ^{13}C NMR spectrum while the attached diastereotopic protons have very similar chemical shifts. In order to test the new method in this situation, a racemic mixture of (2*S*,3*S*)- and (2*R*,3*R*)-N-benzoyl[2,3-$^{2}H_2$]phenylalanine methyl esters (**130**) was prepared by Dr. Paul Reese using literature procedures.[241,242] Figure 18 shows the normal and selective 2-D $^{1}H,^{13}C$ heteronuclear shift correlated spectra of the C-3 region of unlabelled material **131**, a mixture of **130** and unlabelled material **131**, and a mixture of all stereoisomers of [3-^{2}H]phenylalanine (**132**) (generated by enolization and quenching of **130**) and **131**.

In the normal ^{1}H NMR spectrum the two diastereotopic protons have a chemical shift difference of 0.09 ppm. Examination of figure 18 shows that these two multiplets are

clearly resolved in the correlation. In addition to this, the stereospecific labelling of the C-3 position with deuterium in **130** is evident by the appearance of a new signal corresponding to only one of the doublet of doublets of the ABX system. The mixture of all stereoisomers **132** shows α-isotope shifted correlation signals corresponding to both protons of the ABX system.

It is interesting to note that there is a small upfield shift in the position of the deuterated species that can be seen on the vertical proton chemical shift axis. β-isotope shifts by deuterium on protons are known,[243] and appear to be a general phenomenon that is easily observed using this technique.

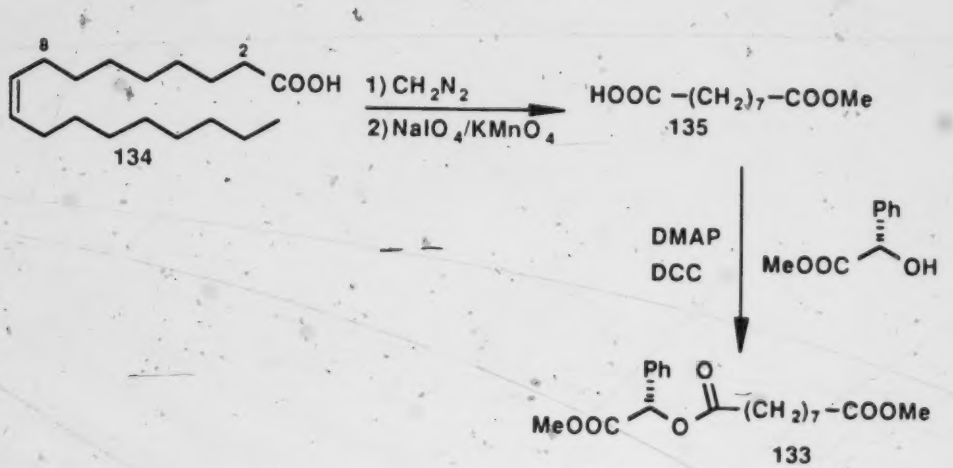
Figure 18. Normal (CH_n) and selective (CH) proton/carbon correlations (deuterium decoupled) of the C-3 region of N-benzoylphenylalanine methyl esters. The corresponding ^1H NMR of **135** is shown on each vertical axis (3.12 - 3.36 ppm).



S-Mandelate ester of monomethyl azelaic acid **133**

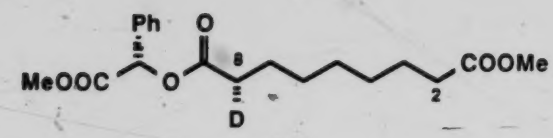
In order to test this technique in a biosynthetic application, *Cladosporium cladosporioides* was incubated with sodium [2-^{13}C,$^{2}H_3$]acetate by Dr. Paul Reese. Oleic acid (**134**) was isolated from the fermentation and methylated with diazomethane (scheme 43). The methyl ester was expected to be chirally deuterated at the C-2 position (refer to figure 12). The ^{13}C NMR spectrum of this compound showed no detectable

Scheme 43.



deuterium induced α-isotope shift at the C-2 carbon. However, C-8 showed the presence of one deuterium. To determine the stereochemistry of deuterium substitution at C-8, methylated **134** was oxidatively cleaved to give monomethyl azelaic acid (**135**). Compound **135** was esterified with *S*-mandelic acid methyl ester using dimethylaminopyridine (DMAP) and 1,3-dicyclohexylcarbodiimide (DCC) to give **133**.

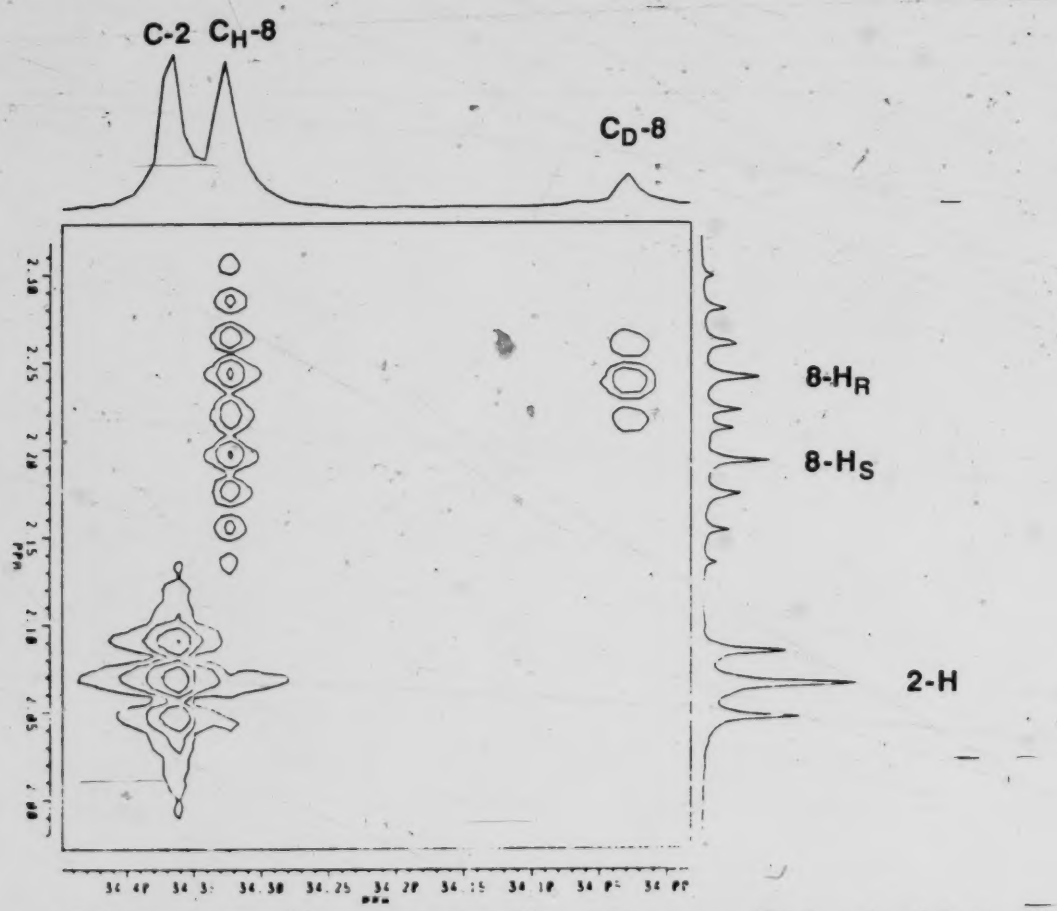
Parker has shown[244] that the pro-*R* hydrogen α to a mandelate ester generally resonates at lower field than does the pro-*S* hydrogen. Examination of the deuterium decoupled ^{1}H,^{13}C heteronuclear shift correlation spectrum of **133** (figure 19) shows clearly that this derivative is labelled in the pro-*S* position by sodium [2-^{13}C,$^{2}H_3$]acetate.



133

The correlation arising from the triplet at 2.07 ppm and its directly bonded carbon at 34.36 ppm is due to the methylene adjacent to the methyl ester and does not interfere with the determination of stereochemistry.

Figure 19. Deuterium-decoupled proton/carbon correlation of the α-methylenes of **133**.



It is apparent from the spectra presented here that stereospecific deuterium labelling can be easily determined by either the normal or selective correlation techniques. Since the use of a fluorine lock is necessary if complete broad band proton and deuterium decoupling

is to be obtained, the major limitation of this technique is instrumental. The ability of this technique to determine which of two diastereotopic protons of a methylene group is labelled makes it ideal for mechanistic, enzymatic[81] and biosynthetic applications.[245]

Experimental

General

All reactions were done under a positive pressure of dry Ar; those requiring non aqueous conditions were performed using oven-dried glassware which was cooled under Ar. All solvents were distilled before use. All organic layers obtained from extractions were dried over anhydrous Na_2SO_4. The term "*in vacuo*" refers to the removal of solvent on a rotary evaporator followed by evacuation (< 0.05 mm Hg) to constant sample weight. All reactions were monitored by TLC using either UV adsorption, I_2 staining, ninhydrin (amino acids) or bromocresol green spray (acids) for visualization. Commercial thin-layer chromatography (TLC) plates were silica, Merck 60F-254, or Merck RP-8F_{254}S. Silica gel for column chromatography was Merck type 60, 70 - 230 mesh. Flash chromatography was done by the method of Still *et al*[246], with Merck type 60 silica gel, 230 - 420 mesh. Reverse phase medium pressure liquid chromatography (MPLC) was done using Merck type 60H silica gel.

All literature compounds had ^1H NMR, MS and IR spectra consistent with assigned structures. Melting points were determined either on a Thomas Hoover or Buchi apparatus using open capillary tubes and are uncorrected. Nuclear magnetic resonance spectra (NMR) were recorded on Bruker WP - 80, WH - 200, AM - 300, WM - 360 or WH - 400 instruments in the specified deuterated solvent with tetramethylsilane (TMS) as internal standard. Infrared spectra (IR) were determined with a Nicolet 7199 FT- IR spectrometer. Mass spectra (MS) were recorded with an ionizing voltage of 70 eV on an AEI MS-50 instrument for electron impact (EI) ionization and on a MS-12 for chemical ionization (CI). Optical rotations were measured on Perkin-Elmer 241 or 141 polarimeters with a microcell (100 mm, 0.9 mL) or a standard cell (100 mm, 8 mL) respectively.

High pressure liquid chromatography (HPLC) was done using either a Hewlett Packard 1082B or 1090 instruments fitted with a Whatman Partisil M9 10/25 column and UV detector set at 254 nm. All gas chromatography (GC) was performed on a Hewlett

Packard 5890A gas chromatograph fitted with either an Alltech 10 m x 0.53 mm bonded FSOT polyphenylmethylsiloxane (RSL-300) column or a J&W Scientific 15 m x 0.53 mm bonded FSOT DB-17+ column with He as the carrier gas. Compounds were detected using a flame ionization detector.

NMR Studies

NMR spectra of labelled compounds were recorded on a Bruker WH-400 spectrometer. Typically, for ^{18}O labelled compounds, 15 - 75 mg of material was dissolved in 0.4 mL of deuterated chloroform containing TMS (5 mm NMR tube). Spectra were accumulated under the following conditions: 32K data block; 4000 Hz sweep width; 0.24 Hz/data point; 2 s acquisition time; 35° pulse width; zero-filled to 64K and line broadening set to 0.12 Hz prior to Fourier transformation.

Deuterated compounds were dissolved in 0.4 ml of $CDCl_3$ containing TMS and 10 drops of hexafluorobenzene (5 mm tubes). Typical accumulation conditions for one dimensional spectra with broad band deuterium decoupling were: 32K data block; 18000 Hz sweep width; 1 Hz/data point; 1 s acquisition time; 30° pulse width; zero-filled to 64K and line broadening set to 0.12 Hz prior to Fourier transformation.

Heteronuclear proton/carbon shift correlations with broad band deuterium decoupling employed previously described pulse sequences to obtain complete[236] and selective[240] two dimensional plots. For the latter, the variable pulse angle θ was set to 90°. All correlation experiments used: a relaxation delay of 3 s between scans; 1 K FID's for 64 values of t_1; zero filling to 512 points in f_1 (^1H) and 2K in f_2 (^{13}C) unless specified otherwise. In each case a Gaussian window function was applied in both f_1 and f_2 with the line broadening set equal to the number of Hz/pt after zero filling. The Gaussian broadening was set to zero. For camphor (2.36 M solution): f_1 (^1H) = 1060 Hz; f_2 (^{13}C) = 5000 Hz; acquisition time 0.10 s; 4 scans/t_1 increment. For N-benzoyl-phenyl-alanine methyl esters (1.29 M solution): f_1 (1H) = 800 Hz; f_2 (^{13}C) = 1800 Hz;

acquisition time = 0.28 s; 8 scans/t_1 increment. For Methyl ((S) Methyl mandelate) nonanedioate (**133**) (0.21 M solution): f_1 (^{1}H) = 600 Hz; f_2 (^{13}C) = 1300 Hz; acquisition time = 0.79 s; 128 scans/t_1 increment; 128 t_1 increments.

Production and Isolation of Cyclosporine (1)

Materials:

Modified literature procedures were employed.[15] The producing organism, *Tolypocladium inflatum*, was obtained from the American Type Culture Collection and is designated No. ATCC 34921. For inoculum propagation, sterilized agar slants were used which contained: malt extract, 10 g; yeast extract, 2 g; agar, 10 g; and 500 mL of distilled water . The production medium contained: sucrose, 50 g; malic acid, 8 g; KH_2PO_4, 0.75 g; $MgSO_4$, 0.5 g; $CaCl_2$, 0.1 g; trace element solution, 1 mL; vitamin solution, 1 mL; and 1 L of distilled water. The media was then brought to pH 4.5 with NH_4OH. The trace element solution consisted of: $ZnSO_4 \cdot 7H_2O$, 4.4 g; $MnCl_2 \cdot 4H_2O$, 180 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 20 mg; $CuSO_4 \cdot 5H_2O$, 80 mg; $FeSO_4 \cdot 7H_2O$, 5.0 g; 2 mL H_2SO_4, and 1 L of distilled water. The vitamin solution consisted of 10 mg each of thiamin and pyridoxine, and 0.5 mg of biotin per litre of distilled water. This was sterilized by membrane filtration.

Cultivation:

Five 250 mL Erlenmeyer flasks containing 50 mL each of the production medium were sterilized (121 °C, 20 min) and inoculated with 1 mL of a suspension of spores from 14 day old agar slants in sterile distilled water (5mL). The flasks were then incubated on a rotary shaker at 180 rpm and 28 °C for 5 days to produce the preculture. Five mL of this preculture were added to each of 10 flasks containing 50 mL of sterilized (121 °C, 20 min) production medium. The production flasks were then incubated on a rotary shaker at 180 rpm and 28 °C for 14 days.

Isolation:

The creamy yellow fermentation broth (500 mL) was rapidly stirred for 24 h with ethyl acetate (600 ml). The mixture was filtered through cheese cloth and the mycelial residue was washed with ethyl acetate (100 ml). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (2 x 500 mL). The combined organic phases were dried and concentrated *in vacuo* to afford 1.14 g of a brown oil. The oil was further purified by flash chromatography[246] (methanol 6%/dichloromethane 94%) and the fractions that contained cyclosporine (**1**) were combined and concentrated *in vacuo* to produce 56.2 mg of a pale gold oil. This oil was purified by gel filtration chromatography on Sephadex LH-20 (2.5 x 60 cm) with methanol to afford 37.0 mg (30.8μmol) of cyclosporine (**1**) as a crystalline white solid: mp 141 - 144 °C, lit.[11] mp 148 - 151 °C; IR ($CHCl_3$ cast) 3317 br, 2956, 1631 br,1470 m, 1411, 1096 m, 753 w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, 8.7 Hz, 1H, NH), 7.65 (d, 8.6 Hz, 1H, NH), 7.48 (d, 8.7 Hz, 1H, NH), 7.17 (d, 8.7 Hz, 1H, NH), 5.70, (m, 1H, HC(α)), 5.48 (d, 5.3 Hz, 1H, HC(α)), 5.35 (m, 3H, CH=CH + HC(α)), 5.14 (d, 11.2 Hz, 1H, HC(α)), 5.10 - 4.94 (m, 3H, HC(α)), 4.84 (p, 7.0 Hz, 1H, HC(α)), 4.74 (d, 12.7 Hz, 1H, COCHHN), 4.66 (t, 9.2 Hz, 1H, HC(α)), 4.53 (p, 7.2 Hz, 1H, HC(α)), 3.80 (br s, 2H, OH + CHOH), 3.52 (s, 3H, NMe), 3.41 (s, 3H, NMe), 3.27 (s, 3H, NMe), 3.15 (d, 12.7 Hz, 1H, COCHHN), 3.13 (s, 3H, NMe), 2.72 (s, 3H, NMe), 2.70 (s, 3H, NMe), 2.50 - 0.80 (m, 69 H), 0.71 (d, 7.9 Hz, 3H, CHOHCHMe); MS 1201 (EI, M^+); MS 1202 (Sulfolane + chloroform, FAB MS, MH^+).

Dihydrocyclosporine (2)[11]

The procedure of Ruegger *et al* was employed[11]. A suspension of cyclosporine (**1**) (14.2 mg, 11.8μmol) and 5% Pd/C (1.2 mg) in 1 mL of absolute ethanol was stirred under an atmosphere of H_2 for 12 h. The catalyst was removed by filtration and washed with absolute ethanol (2 x 1 mL). The combined organic phases were concentrated *in*

vacuo to give a colourless glass that was purified on an analytical TLC plate (10 x 20 cm, chloroform 94%/methanol 6%) to afford 9.93 mg of **2** (70% yield) as a colourless glass: mp 149 - 151 °C, lit.[11] mp 151 - 153 °C; IR ($CHCl_3$ cast) 3320, 2959, 1631, 1470, 1411, 1094, 753 cm^{-1}; ^1H partial NMR (200 MHz, $CDCl_3$) δ 5.35 (dd, 11.2, 3.7 Hz, 1H, HC(α)), 2.50 - 0.80 (m, 73 H), all other peaks are identical to **1**; MS 1203 (EI, M^+).

Dideuterocyclosporine (3)

The procedure of Ruegger *et al*[11] was used to produce **3** (49.1 mg, 98% yield) as a crystalline solid from 50.0 mg of cyclosporine (**1**) (42 μmol) and deuterium gas: mp 150 - 154 °C, lit.[11] mp 151 - 153 °C; IR ($CHCl_3$ cast) 3320, 2959, 1627, 1470, 1408, 1095, 753 cm^{-1}; ^1H partial NMR (200 MHz, $CDCl_3$) δ 5.35 (dd, 11.2, 3.7 Hz, 1H, HC(α)), 2.50 - 0.80 (m, 71H), all other peaks are identical to **1**; MS 1205 (EI, M^+).

Ditritiocyclosporine (4)

Modified literature procedures were employed[11]. Hydrogenation with tritium gas was carried out by Amersham on 19.1 mg of cyclosporine (**1**) (15.9 μmol) in ethanol using 5% Pd/C as catalyst. The crude sample in ethanol (140 mL) was returned to our labs for purification. The radioactive ethanol was removed by lyophilization and the sample was lyophillized again from absolute ethanol (2 x 140 mL). Purification of the crude tritiated cyclosporine by column chromatography (5 g silica gel, chloroform 96%/methanol 4%) produced 17.2 mg (90% yield) of **4** that was pure by TLC using both iodine and a Berthold LB 2760 radiochromatogram TLC-scanner for visualization. ^1H partial NMR (400 MHz, $CDCl_3$) δ 5.35 (dd, 11.2, 3.7 Hz, 1H, HC(α)), all other peaks are identical to **1**. ^3H activity was 40 Ci/mmol.

Iodocyclosporine (6)[11]

The procedure of Ruegger *et al*[11] was used. To a stirred solution of cyclosporine (**1**) (10.6 mg, 8.8 µmol) and thallium (I) acetate (3.2 mg, 12 µmol) in 0.75 mL chloroform was added a solution of iodine (3.1 mg, 12 µmol) in 0.75 mL of chloroform. The reaction was stirred in the dark for 16 h, filtered, concentrated *in vacuo*, and purified on an analytical TLC plate (20 x 10 cm, chloroform 96%/methanol 4%) to give 6.8 mg of **7** (58% yield) as a crystalline solid: mp 149 - 151 °C dec, lit.[11] mp 150 °C dec; IR ($CHCl_3$ cast) 3320, 2959, 1630, 1472, 1409, 1097, 753 cm^{-1}; ^1H partial NMR (400 MHz, $CDCl_3$) δ 5.35 (m, 1H, HC(α)), 3.97 (m, 2H, CHOCH), all other peaks are identical to **1**; MS 1328 (Cleland FAB MS, M^+).

Bromocyclosporine (7)

The conditions of Demole *et al*[29] were adapted. A solution of cyclosporine (**1**) (50 mg, 42 µmol) and N-bromosuccinimide (9.0 mg, 50µmol) in carbon tetrachloride (1 mL) was stirred for 24 hours. The solvent was then removed *in vacuo* to give an oil. Upon purification by flash chromatography[246] (chloroform 98%/methanol 2%) 21 mg of **8** (40% yield) was obtained as a crystalline solid: mp 163 °C dec.; IR ($CHCl_3$ cast) 3315, 2958, 1630, 1470, 1383, 1083 cm^{-1}; ^1H partial NMR (400 MHz, $CDCl_3$) δ 5.35 (m, 1H, HC(α)), 3.92 (m, 2H, CHOCH), all other peaks are identical to **1**; MS 1279 (EI, M^+).

O-Acylcyclosporine (11)[14]

The procedure of Traber *et al*[14] was employed. A solution of **1** (100 mg, 83 µmol) and N,N-dimethylaminopyridine (15.0 mg, 120 µmol) in 2 mL acetic anhydride was stirred for 20 h, and the solvent was removed *in vacuo* to give a pale yellow oil. The oil was dissolved in chloroform (2 mL), washed with 0.1 N HCl (2 x 2 mL) and water (2 mL), dried and concentrated *in vacuo* to give a yellow crystalline solid. The solid was purified by flash chromatography[14] (chloroform 98%/methanol 2%) to afford 97 mg of **11**

(94% yield) as a white crystalline solid: mp 228 - 230 °C, lit.[14] mp 230 - 231 °C; IR ($CHCl_3$ cast) 3320, 2959, 1744, 1627, 1470, 1411, 1232, 1096, 753 cm^{-1}; ^1H partial NMR (200 MHz, $CDCl_3$) δ 2.03 (s, 3H, COMe), all other peaks are identical to **1**; MS 1243 (EI, M^+).

Isocyclosporine (14)[11]

The literature procedure of Rugger *et al*[11] was used. To a stirred solution of **1** (30.0 mg, 25 μmol) in methanol (1 mL) was added 13 μL of methanesulfonic acid (200 μmol). The solution was then stirred for 48 h at 50 °C, cooled to room temperature, and sodium acetate (25 mg) was added. The solid was removed by filtration, the solution was concentrated *in vacuo*, and the residue was purified by preparative TLC (20 x 20 x 0.2 cm, chloroform 90%/methanol 6%/acetic acid 4%) to give 15.4 mg of **14** (51% yield) as a colourless crystalline solid: mp 142 - 143 °C, lit.[11] mp 142 - 144 °C, IR ($CHCl_3$ cast) 3321, 2960, 1738, 1642, 1480, 1409, 1097 cm^{-1}; ^1H partial NMR (400 MHz, $CDCl_3$) δ 3.50 (s, 3H, NMe), 3.41 (s, 3H, NMe), 3.16 (d, 12.7Hz, 1H, COCHHN), 3.13 (s, 6H, NMe), 3.10 (s, 3H, NMe), 2.72 (s, 3H, NMe), 2.70 (s, 3H, NMe), all other peaks are identical to **1**; MS 1201 (EI, M^+).

^1H NMR Studies of Cyclosporine Metabolite (17)[41]

The ^1H NMR measurements were done on a Bruker WM - 360 spectrometer using 0.7 mg of metabolite in $CDCl_3$ (0.4 mL). Spectra were obtained using a 32K data block, 3.28 s acquisition time, 5000 Hz sweep width, 36° pulse angle and 256 scans: ^1H partial NMR (360 MHz, $CDCl_3$) δ 7.01 (dd, 15.6, 7.5 Hz, 1H, CH=CHCOOH), 5.79 (d, 15.1 Hz, 1H, CH=CHCOOH), 5.67 (m, 1H, HC(α)), all other peaks are identical to **1**.

[4*R*,5*S*]-4-Methyl-5-phenyloxazolidin-2-one (22)[100]

To a solution of triethylamine (44.1 mL, 153 mmol) in dichloromethane (300 mL) was added (+)-norephedrine hydrochloride (28.2 g, 150 mmol). The mixture was cooled to 0 °C, phenylchloroformate was added dropwise and the mixture was then allowed to warm to room temperature. Triethylamine (132 mL, 459 mmol) was added and the mixture was heated to reflux overnight. The cooled solution was washed with 5% Na_2CO_3 (3 x 100 mL), 1N HCl (3 x 100 mL), and water (2 x 100 mL). The organic layer was dried and concentrated *in vacuo* to give a golden oil that was purified by flash chromatography[246] (ethyl acetate) to afford 14.3 g of **22** (53% yield) as a crystalline white solid: $[\alpha]_D$ = + 161° (c 1.0, $CHCl_3$), lit.[100] $[\alpha]_D$ = +163.7° (c 1.0, $CHCl_3$); mp 118 - 119 °C, lit.[100] mp 120 - 121 °C; IR ($CHCl_3$ cast) 3290 br, 1753, 1387, 1245, 949, 703 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.26 (m, 5H, Ph), 6.75 (br s, 1H, NH), 5.65 (d, 8.1 Hz, 1H, PhCH), 4.19 (m, 1H, HCCH$_3$), 0.78 (d, 6.8 Hz, 3H, CH$_3$); exact mass 177.0793 (177. 0790 calculated for $C_{10}H_{11}NO_2$). Anal. Calc. for $C_{10}H_{11}NO_2$: C, 67.78; H, 6.26; N, 7.90. Found: C, 67.67; H, 6.28; N, 7.89.

[4*R*,5*S*]-3-(1-Oxohexyl)-4-methyl-5-phenyloxazolidin-2-one (24)

The procedure of Evans *et al*[100] was adapted to convert hexanoyl chloride (0.96 g, 7.1 mmol) and the oxazolidinone **22** (1.04 g, 5.9 mmol) to 1.45 g (89% yield) of **24** as an oil. This compound exists as a mixture of rotamers: IR ($CHCl_3$ cast) 2955, 1785, 1703, 1350, 1197, 765, 700 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.25 (m, 5H, Ph), 5.65 (d, 6.8 Hz, 0.7H, PhCHO), 5.05 (d, 4.0 Hz, 0.3H, PhCHO), 4.74 (m, 0.7H, CH$_3$CHN), 4.39 (m, 0.3H, CH$_3$CHN), 2.93 (m, 2H, COCH$_2$), 1.68 (m, 2H, COCH$_2$CH$_2$), 1.55 (d, 6.0 Hz, 0.9H, NCHCH$_3$), 1.34 (m, 4H, CH$_2$CH$_2$CH$_3$), 0.90 (m, 5.1H, CH$_2$CH$_3$ + NCHCH$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 172.5, 152.7, 152.6, 137.3, 133.4, 128.7, 128.2, 125.3, 125.0, 81.2, 78.5, 57.6, 54.2, 35.3, 35.1, 30.9, 30.8, 23.7, 22.0,

18.9, 13.5; exact mass 275.1516 (275.1521 calculated for $C_{16}H_{21}NO_3$). Anal. Calc. for $C_{16}H_{21}NO_3$: C, 69.84; H, 7.69; N, 5.09. Found: C, 69.79; H, 7.68; N, 5.00.

[3(2*R*),4*R*,5*S*]-3-(1-Oxo-2-methylhexyl)-4-methyl-5-phenyloxazolidin-2-one (25)

The procedure of Evans *et al* was employed.[99] To a stirred solution of carboximide **24** (1.45 g, 5.3 mmol) in THF (15 mL) at -78 °C was added a solution of lithium hexamethylsilazide (9.8 mL, 0.59 M, 5.8 mmol). The mixture was warmed after 30 min to 0 °C, methyl iodide (0.98 mL, 15.8 mmol) was added and the solution was stirred for an additional 4 h. Excess solvent was removed *in vacuo* and the residue was purified by flash chromatography[246] (dichloromethane 50%/hexane 50%) to give 1.23 g of **25** (81% yield) as a colourless oil. This compound exists as a mixture of rotamers: IR (neat film) 2930, 1785, 1700, 1455, 1342, 1195, 1121, 958, 698 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.26 (m, 5H, Ph), 5.58 (d, 7.2 Hz, 0.8H, PhCH), 4.99 (d, 4.1 Hz, 0.2H, PhCH), 4.69 (m, 0.8H, $PhCHCHCH_3$), 4.36 (m, 0.2H, $PhCHCHCH_3$), 3.64 (m, 1H, $COCHCH_3CH_2$), 1.46 (d, 8.0 Hz, 0.8H, $PhCHCHCH_3$), 1.40 (m, 6H, $(CH_2)_3$), 1.08 (d, 8.8 Hz, 3H, $COCHCH_3CH_2$), 0.78 (m, 5.2H, $PhCHCHCH_3$ + $CH_2CH_2CH_3$); exact mass 289.1676 (289.1678 calculated for $C_{17}H_{23}NO_3$). Anal. Calc. for $C_{17}H_{23}NO_3$: C, 70.56; H, 8.01; N, 4.84. Found: C, 70.29; H, 8.07; N, 4.70.

Large Scale Preparation of 25 and Isolation of [3(2*S*),4*R*,5*S*]-3-(1-Oxo-2-methylhexyl)-4-methyl-5-phenyloxazolidin-2-one (26)

The method of Evans *et al* was used.[99] To a solution of carboximide **24** (14.6 g, 53 mmol) in THF (150 mL) at -78 °C was added a solution of sodium hexamethylsilazide (96 mL, 0.55 M, 53 mmol). After 30 min the mixture was warmed to 0 °C, methyl iodide (10.0 mL, 160 mmol) was added and the reaction stirred for an additional 3 h. Excess

solvent was removed *in vacuo* and the residue purified by flash chromatography[246] (ethyl acetate 14%/hexane 86%) to give 6.17 g (40% yield) of the [3(2*R*),4*R*,5*S*] isomer **25** whose physical constants were identical to those described above. In addition, 5.82 g (38% yield) of the [3(2*S*),4*R*,5*S*] isomer **26** was isolated as a colourless oil. This compound exists as a mixture of rotamers: IR (neat film) 2930, 1783, 1701, 1458, 1367, 1192, 1125, 959, 697 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.38 (m, 5H, Ph), 5.66 (d, 7.2 Hz, 0.1H, $PhCHCHCH_3$), 5.08 (d, 4.5 Hz, 0.9H, $PhCHCHCH_3$), 4.80 (m, 0.1H, $PhCHCHCH_3$), 4.44 (m, 0.9H, $PhCHCHCH_3$), 3.78 (m, 1H, $COCHCH_3CH_2$), 1.70 (m, 2H, $COCHCH_3CH_2$), 1.54 (d, 5.6 Hz, 3H, $PhCHCHCH_3$), 1.28 (m, 4H, $(CH_2)_2CH_3$), 1.12 (d, 6.9 Hz, 3H, $COCHCH_3CH_2$), 0.87 (t, 6.8 Hz, 3H, $CH_2CH_2CH_3$); exact mass 289.1681 (289.1678 calculated for $C_{17}H_{23}NO_3$). Anal. Calc. for $C_{17}H_{23}NO_3$: C, 70.56; H, 8.01; N, 4.84. Found: C, 70.42; H, 7.96; N, 4.78.

(*S*)-2-Amino-3-methylbutan-1-ol (28)[248]

A modification of the procedure of Hsuno *et al*[102] was followed. A mixture of lithium aluminium hydride (30 g, 0.79 mol) and THF (1.5 L) was heated to reflux under argon for 30 min. The mixture was cooled to 0 °C and L-valine (70 g, 0.60 mol) was added in small portions. Heating under reflux was continued for 17 h, celite (40 g) was then added, followed by water (40 mL), 10% aqueous sodium hydroxide (40 mL), and water (120 mL). The mixture was filtered and the solid was washed with ethyl acetate (2 x 200 mL). The combined filtrates were concentrated *in vacuo* and the residue was distilled under reduced pressure to give 52.1 g (84%) of **28** as a colourless oil: bp 56 - 58 °C at 0.5 mm Hg, lit.[247] bp 55 - 57 °C at 2 mm Hg; $[\alpha]_D$ = + 14.0° (neat), lit.[247] $[\alpha]_D$ = + 14.6° (neat); IR ($CHCl_3$ cast) 3288 br, 2960 br, 1590, 1468, 1337, 1319, 1054 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 3.64 (dd, 10.4, 4.1 Hz, 1H, $OCHHCHN$), 3.40 (dd, 10.4, 8.0 Hz, 1H, $OCHHCHN$), 2.57 (m, 1H, $OCHHCHN$), 2.36 (br s, 3H, $OH + NH_2$), 1.57 (m,

1H, $CH(CH_3)_2$), 0.95 (d, 6.8 Hz, 6H, $CH(CH_3)_2$); exact mass 104.1085 (104.1075 calculated for $C_5H_{14}NO$, MH^+).

(*S*)-4-Isopropyloxazolidin-2-one (29)[100]

A modification to the procedure of Newman *et al*[101] was used. To a stirred solution of **28** (10 g, 97 mmol) in toluene (300 mL) at 0 °C was added phosgene (7.0 mL, 110 mmol) by distillation. Triethylamine (13.5 mL, 110 mmol) was added dropwise to the resulting white slurry and the mixture was refluxed for 20 min. The triethylamine hydrochloride was removed by filtration after cooling and the filtrate was concentrated *in vacuo* to give 15.4 g of an orange liquid that crystallized on standing. The product was purified by flash chromatography[246] (hexane 60%/ethyl acetate 40%) and recrystallized from hexane/ethyl acetate to give 9.71 g (77% yield) of **29** as fine white crystals: $[\alpha]_D$ = +14.4° (c 7.1, $CHCl_3$), lit.[100] $[\alpha]_D$ = +14.8° (c 7.0, $CHCl_3$); mp 71 - 72 °C, lit.[100] mp 71 - 72 °C; IR ($CHCl_3$ cast) 3260 br, 2960, 1746, 1248, 1092, 937, 710 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.91 (br s, 1H, NH), 4.46 (t, 8.8 Hz, 1H, OCHHCHN), 4.13 (dd, 8.8, 7.3 Hz, 1H, OCHHCHN), 3.62 (q, 7.9 Hz, 1H, OCHHCHN), 1.75 (m, 1H, $CH(CH_3)_2$), 0.96 (d, 6.9 Hz, 3H, $CH(CH_3)_2$), 0.91 (d, 6.8 Hz, 3H, $CH(CH_3)_2$); exact mass 129.0790 (129.0790 calculated for $C_6H_{11}NO_2$).

(*R*)-4-Isopropyloxazolidin-2-one (30)[100]

The procedure used to transform **28** into **29** was employed to convert **31** (10 g, 97 mmol) to 9.83 g of **30** (78% yield): $[\alpha]_D$ = -15.1° (c 7.0, $CHCl_3$), lit.[100] $[\alpha]_D$ = +14.8° (c 7.0, $CHCl_3$) for the *S*-isomer; mp 72 - 72 °C, lit.[100] mp 71 - 72 °C; all other physical constants were identical to those of **29**.

(*R*)-2-Amino-3-methylbutan-1-ol (31)[247]

The procedure used to transform L-valine into **28** was employed to convert D-valine (35.0 g, 0.3 mol) to 18.3 g (60% yield) of **31** as a colourless oil: bp 51 - 53 °C at 0.25 mm Hg, lit.[247] bp 55 - 57 °C at 2 mm Hg; $[\alpha]_D$ = -14.7° (neat), lit.[247] $[\alpha]_D$ = +14.6 (neat) for the *S*-isomer; all other physical constants were identical to those of **28**.

(*S*)-3-(1-Oxo-3-phenylpropyl)-4-isopropyloxazolidin-2-one (32)[96]

The procedure of Evans *et al*[100] was adapted to convert dihydrocinnamoyl chloride (5.75 mL, 38.7 mmol) and the oxazolidinone **29** (5.00 g, 38.7 mmol) to 9.03 g (89% yield) of **32**: mp 75 - 76 °C, lit.[96] mp 63 - 64 °C; IR ($CHCl_3$ cast) 2955, 1794, 1772, 1706, 1305, 1211 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.28 (m, 5H, Ph), 4.45 (m, 1H, OCHHCHN), 4.17 (m, 2H, OCHHCHN), 3.25 (m, 2H, $COCH_2$), 2.95 (m, 2H, CH_2Ph), 2.38 (m, 1H, $CH(CH_3)_2$), 0.85 (d, 7.2 Hz, 3H, $CH(CH_3)_2$), 0.79 (d, 7.0 Hz, 3H, $CH(CH_3)_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.5, 153.5, 140.2, 127.6, 127.4, 125.3, 62.8, 57.4, 36.4, 29.7, 27.2, 16.7, 13.6; exact mass 261.1364 (261.1365 calculated for $C_{15}H_{19}NO_3$). Anal. Calc. for $C_{15}H_{19}NO_3$: C, 68.95; H, 7.33; N, 5.36. Found: C, 69.20; H, 7.51; N, 5.32.

[3(2*S*),4*S*]-3-(1-Oxo-2-methyl-3-phenylpropyl)-4-isopropyloxaxolidin-2-one (33)

The method of Evans *et al*[99] was used. To a cooled (-78 °C) solution of carboximide **32** (1.00 g, 3.83 mmol) in THF (8 mL) was added a solution of LDA (4.4 mL, 0.86 M, 3.83 mmol). The solution was stirred for 10 min, warmed to 0 °C, and methyl iodide (0.71 mL, 11.5 mmol) was added. Aqueous NH_4Cl (5%, 10 mL) was added followed by 30 mL water and the mixture was extracted with dichloromethane (3 x 15 mL). The combined organic phases were washed with 5% $NaHCO_3$ (10 mL), and brine (10 mL), dried, and concentrated *in vacuo* to give 1.08 g of a golden oil. Purification

of the oil by flash chromatography[246] (ethyl acetate 25%/ hexane 75%) afforded 0.81 g (77% yield) of **33** as a colourless oil: IR ($CHCl_3$ cast) 2963, 1780, 1700, 1387, 1301, 1237, 1204, 700 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.26 (m, 5H, Ph), 4.27 (m, 1H, OCHHCHN), 4.06 (m, 3H, OCHHCHN + $COCHCH_3CH_2Ph$), 3.02 (dd, 13.2, 7.2 Hz, 1H, CHHPh), 2.56 (dd, 13.4, 6.8 Hz, 1H, CHHPh), 2.32 (m, 1H, $CH(CH_3)_2$), 1.24 (d, 7.0 Hz, 3H, $COCHCH_3CH_2Ph$), 0.89 (d, 7.0 Hz, 3H, $CH(CH_3)_2$), 0.85 (d, 7.0 Hz, 3H, $CH(CH_3)_2$); exact mass 275.1516 (275.1521 calculated for $C_{16}H_{21}NO_3$). Anal. Calc. for $C_{16}H_{21}NO_3$: C, 69.80; H, 7.69; N, 5.09. Found: C, 69.88; H, 7.63; N, 4.86.

4,5-Diphenyl-1,3-dioxol-2-one[103] (35)

The procedure of Sheehan and Guziec[103] was followed. To a stirred slurry of benzoin (17.0 g, 80 mmol) in benzene (100 mL) at 0 °C was added phosgene (6.4 mL, 88 mmol) by distillation. Dimethylaniline (10.2 mL, 80 mmol) was added dropwise over a period of 30 min, and the solution was warmed to room temperature and stirred for 12 h. The dimethylaniline hydrochloride was removed by filtration. The solution was refluxed for 3 h, washed with 0.5 N HCl (60 mL), and water (60 mL), dried, and concentrated *in vacuo* to give a green oil. The oil was crystallized from the minimal amount of 95% ethanol to produce a solid that was recrystallized from ethanol/water to afford 10.3 g of **35** (54% yield) as white needles: mp 71 - 72 °C, lit.[103] mp 75 - 76 °C; IR ($CHCl_3$ cast) 3060, 1820, 1450, 1210, 1027, 760 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.61 (m, 4H, Ph), 7.48 (m, 6H, Ph); exact mass 238.0632 (238.0630 calculated for $C_{15}H_{10}O_3$).

2-(2-Oxo-4,5-diphenyl-1,3-oxazol-3-yl)acetic acid[103] (36)

The method of Sheehan and Guziec[103] was employed. Glycine (1.26 g, 16.8 mmol) was dissolved in a solution of tetramethylammonium hydroxide (20% in methanol, 7.66 mL, 16.8 mmol) and the solvent was removed under reduced pressure. The residue

was dissolved in DMF (16 mL), dioxolone **35** (4.00 g, 1.68 mmol) was added, and the mixture was stirred at room temperature for 30 min. The mixture was then acidified by the addition of 2N HCl (16 mL) and extracted with ethyl acetate (2 x 40 mL). The combined organic phases were washed with water (3 x 50 mL), dried, and concentrated *in vacuo* to afford a white solid. Recrystallization of the solid from ethyl acetate/hexane gave 3.98 g (80% yield) of **36** as a white crystalline solid: mp 178 - 179 °C, lit.[103] mp 178 - 179 °C; IR ($CHCl_3$ cast) 3100 br, 1768, 1745, 1487, 1188, 765 cm^{-1}; ^1H NMR (80 MHz, DMSO-d_6) δ 8.22 (s, 1H, COOH), 7.41 (m, 6H, Ph), 7.30 (m, 4H, Ph), 4.00 (s, 2H, CH_2); exact mass 295.0846 (295.0844 calculated for $C_{17}H_{13}NO_4$).

(*S*) 3-(1-Oxo-2-[2-oxo-4,5-diphenyl-1,3-oxazol-3-yl]ethyl)-4-isopropyloxazolidin-2-one (38)

A solution of **36** (3.50 g, 11.9 mmol) in thionyl chloride (20 mL) was heated to reflux for 3 h, cooled to room temperature, and concentrated *in vacuo* . The residue was dissolved in THF (5 mL) and added to a cooled (0 °C) solution of oxazolidinone **29** (1.50 g, 11.6 mmol), n-butyllithium (1.6 M in hexane, 7.3 mL, 11.6 mmol), and THF (50 mL). The reaction was stirred for 30 min, quenched by the addition of 5% Na_2CO_3 (20 mL), and extracted with dichloromethane (3 x 50 mL). The combined organic phases were dried and concentrated *in vacuo* to give 4.20 g of an orange oil. Purification by silica gel chromatography (250 g silica, ethyl acetate 60%/hexane 40%), gave 1.82 g (40% yield) of **38** as a foam: IR ($CHCl_3$ cast) 2985, 1762, 1710, 1404, 1268, 1075, 767 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.51 (m, 3H, Ph), 7.42 (m, 2H, Ph), 7.31 (m, 2H, Ph), 7.25 (m, 3H, Ph), 4.91 (d, 17.6 Hz, 1H, COC**H**HN), 4.76 (d, 17.5 Hz, 1H, COCH**H**N), 4.38 (m, 1H, OC**H**HCHN), 4.34 (t, 8.8 Hz, 1H, OCH**H**CHN), 4.25 (dd, 8.8, 3.6 Hz, 1H, OCHHC**H**N), 2.29 (m, 1H, C**H**$(CH_3)_2$), 0.88 (d, 6.7Hz, 3H, CH(C**H**$_3)_2$), 0.74 (d, 6.7 Hz, 3H, CH(C**H**$_3)_2$); exact mass 406.1535 (406.1528 calculated for $C_{23}H_{22}N_2O_5$).

Anal. Calc. for $C_{23}H_{22}N_2O_5$: C, 67.97; H, 5.46; N, 6.89. Found: C, 68.02; H, 5.51; N, 6.60.

Deuteration of 38. (*S*) 3-(1-Oxo-2-deutero-2-[2-oxo-4,5-diphenyl-1,3-oxazol-3-yl]ethyl)-4-isopropyloxazolidin-2-one (40)

To a solution of **38** (200 mg, 0.49 mmol) in THF (1.63 mL) at -78 °C was added a solution of LDA in THF (0.8 mL, 0.63 M, 0.50 mmol). The reaction was stirred for 5 min, warmed to 0 °C, quenched with trifluoroacetic acid-d_1 (38 μL, 0.50 mmol), and taken to dryness *in vacuo* . The residue was purified by silica gel chromatography (2 g silica, ethyl acetate) to give 1.87 mg of **40** (93% yield) as a yellow foam: ^{1}H partial NMR (200 MHz, $CDCl_3$) δ 4.91 (d, 17.6 Hz, 0.45H, COCHHN), 4.76 (d, 17.6 Hz, 0.45H, COCHHN); exact mass 408.1653 (408.1652 calculated for $C_{23}{}^1H_{20}{}^2H_2N_2O_5$, relative intensity 22%), 407.1605 (407.1590 calculated for $C_{23}{}^1H_{21}{}^2H_1N_2O_5$, relative intensity 38%), 406.1546 (406.1528 calculated for $C_{23}{}^1H_{22}N_2O_5$, relative intensity 100 %).

(*S*)-3-(1-Oxo-3-methylbutyl)-4-isopropyloxazolidin-2-one (52)

The method of Evans *et al*[100] was adapted to convert isovaleryl chloride (0.61 mL, 5.0 mmol) and oxazolidinone **29** (0.65 g, 5 mmol) to 0.98 g (92% yield) of **52** as a colourless oil: IR ($CHCl_3$ cast) 2962, 1784, 1701, 1389, 1306, 1024 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$) δ 4.35 (m, 3H, OCHHCHN), 2.83 (m, 2H, $COCH_2$), 2.28 (m, 2H, $CH(CH_3)_2$), 0.95 (m, 12H, $CH(CH_3)_2$); exact mass 213.1363 (213.1365 calculated for $C_{11}H_{19}NO_3$). Anal. Calc. for $C_{11}H_{19}NO_3$: C, 61.95; H, 8.98; N, 6.57. Found: C, 61.80; H, 8.76; N, 6.51.

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(benzyloxycarbonyl)-hydrazino]-3-methylbutyl)-4-isopropyloxazolidin-2-one (53)

The procedure used to transform **56** to **54** was employed to produce **53** from 615 mg (2.88 mmol) of carboximide **52** and dibenzyl azodiformate. The crude product was purified by flash chromatography[246] (ethyl acetate 40%/hexane 60%) to give 1.26 g of **53** (85% yield) as a colourless oil that crystallized on standing: HPLC diastereomer analysis (22% hexane/78% ethyl acetate, 2 mL/min) showed a ratio of 97 : 3 for the 3(2*S*) (t_r = 30.4 min) and 3(2*R*) (t_r = 25.9 min) isomers; mp 109 - 111 °C; IR ($CHCl_3$ cast) 2960 br, 1782, 1761,1721, 1388, 1216, 1054 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.13 (m, 10H, Ph), 6.91 (br s, 1H, NH), 6.19 (d, 9.6 Hz, 1H, COCHCH), 5.08 (m, 4H, OCH_2Ph), 4.06 (m, 1H, OCHHCHN), 3.65 (dd, 8.8, 3.2 Hz, 1H, OCHHCHN), 3.56 (t, 8.4 Hz, 1H, OCHHCHN), 2.40 (m, 1H, $COCHCH(CH_3)_2$), 2.17 (m, 1H, under toluene signal, $CH(CH_3)_2$), 1.12 (d, 6.5 Hz, 3H, $COCHCH(CH_3)_2$), 1.01 (d, 6.5 Hz, 3H, $COCHCH(CH_3)_2$); 0.64 (d, 6.4 Hz, 3H,CH_2CH $CH(CH_3)_2$), 0.56 (d, 6.3 Hz, 3H, $CH_2CHCH(CH_3)_2$); MS 512 (CI, MH^+). Anal. Calc. for $C_{27}H_{33}N_3O_7$: C, 63.39; H, 6.50; N, 8.21. Found: C, 63.32; H, 6.61; N, 7.98.

General Procedure for Formation of Lithium Enolates of Carboximides and Subsequent Amination with Dialkyl Azodiformates

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(benzyloxycarbonyl)-hydrazino]propyl)-4-isopropyloxazolidin-2-one (54)

The enolate was formed using modified literature procedures.[99] A 0.81 M solution of LDA in THF/hexane (4 mL, 3.2 mmol) was added to a solution of oxazolidinone carboximide **56** (458 mg, 2.5 mmol) in THF (35 mL) at -78 °C. The solution was stirred for 30 min and a solution of dibenzyl azodiformate (945 mg, 3.2 mmol) in THF (2 ml) was added over a period of 5 min. The reaction was then quenched by the addition of aqueous 5% NH_4Cl (20 mL), warmed to room temperature, and extracted with dichloromethane (3

x 75 mL). The combined organic phases were dried and concentrated *in vacuo* to give 1.63 g of an orange oil that solidified upon standing. The solid was purified by flash chromatography[246] (32% ethyl acetate/68% hexane) to afford 1.09 g (91% yield) of **54** as a white amorphous solid: HPLC analysis (46% hexane/46% dichloromethane/8% acetonitrile, 1 mL/min) showed that the ratio of diastereomers was 90:10 (± 2%) with retention times (t_r) of 14.6 and 19.0 min, respectively. The major diastereomer was shown to have the (2*S*) configuration by conversion to L-alanine (see below): mp 116 - 118 °C; IR ($CHCl_3$ cast) 3438 br, 1783, 1755, 1720, 1388, 1300, 1220 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.10 (m, 10H, Ph), 6.82 (br s, 1H, NH), 5.99 (q, 8.8 Hz, 1H, COCHCH$_3$), 5.08 (m, 4H, OCH$_2$Ph), 3.88 (m, 1H, OCHHCHN), 3.59 (dd, 8.7, 5.6 Hz, 1H, OCHHCHN), 3.49 (t, 9.6 Hz, 1H, OCHHCHN), 2.14 (m, 1H, under toluene signal, CH(CH$_3$)$_2$), 1.48 (d, 8.8 Hz, 3H, CHCH$_3$), 0.59 (d, 7.2 Hz, 3H, CH(CH$_3$)$_2$), 0.53 (d, 7.2 Hz, 3H, CH(CH$_3$)$_2$); exact mass 483.2008 (483.2005 calculated for $C_{25}H_{29}N_3O_7$). Anal. Calc. for $C_{25}H_{29}N_3O_7$: C, 62.10; H, 6.05; N, 8.64. Found: C, 62.39; H, 6.19; N, 8.35.

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(benzloxycarbonyl)-hydrazino]-3-phenylpropyl)-4-isopropyloxazolidin-2-one (55)

The procedure used to transform **56** to **54** was employed to produce **55** from 1.00 g (3.83 mmol) of carboximide **32** and dibenzyl azodiformate. The crude product was purified by MPLC (50 g Merck Kieselgel 60H, ethyl acetate 35%/hexane 65%, 3 mL/min) to afford 1.93 g of **55** (90% yield) as a white amorphous solid: HPLC diastereomer analysis (78% hexane/22% ethyl acetate, 1 mL/min) gave a ratio of 94:6 for the 3(2*S*) (t_r = 48.2 min) and 3(2*R*) (tr = 44.7 min) isomers; mp 118 - 119.5 °C; IR ($CHCl_3$ cast) 2868 br, 1782, 1720 br, 1388, 1300, 1216 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.10 (m, 15H, Ph), 6.85 (br s, 1H, NH), 6.47 (t, 7.3 Hz, 1H, CHCH$_2$Ph), 4.98 (m, 4H, OCH$_2$Ph), 3.87 (m, 1H, OCHHCHN), 3.47 (dd, 9.2, 2.7 Hz, 1H, OCHHCHN),

3.30 (d, 9.2 Hz, 1H, OCHHCHN), 3.28 (d, 7.3 Hz, $CHCH_2Ph$), 2.10 (m, 1H, under toluene signal, $CH(CH_3)_2$), 0.58 (d, 7.0 Hz, 3H, $CH(CH_3)_2$), 0.53 (d, 7.0 Hz, 3H, $CH(CH_3)_2$); ^{13}C NMR (100 MHz, toluene-d_8, 374 °K) δ 172.6, 156.5, 156.3, 153.3, 137.4, 137.1, 136.9, 130.2, 129.3, 128.7, 128.6, 128.5, 128.4, 128.3, 128.0, 127.1, 68.7, 67.8, 63.8, 62.3, 59.4, 36.0, 29.2, 17.8, 15.1; exact mass 424.1875 (424.1872 calculated for $C_{23}H_{26}N_3O_5$, $M^+ - C_8H_7O_2$); MS 577 (CI, M + NH_4^+). Anal. Calc. for $C_{31}H_{33}N_3O_7$: C, 66.54; H, 5.94; N, 7.51. Found: C, 66.16; H, 5.84; N, 7.40.

(*S*)-3-(1-Oxopropyl)-4-isopropyloxazolidin-2-one (56)[100]

The procedure of Evans and coworkers[100] was used to convert propionyl chloride (4.8 mL, 55 mmol) and (*S*)-4-isopropyloxaxolidin-2-one (**29**) (7.00 g, 54.2 mmol) to 10.8 g (98%) of **56** as a colourless oil: IR ($CHCl_3$ cast) 2964, 1778, 1704, 1463, 1208, 1071 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.45 (dd, 8.5, 3.6 Hz, 1H, OCHHCHN), 4.31 (t, 8.5 Hz, 1H, OCHHCHN), 4.26 (dd, 8.7, 3.2 Hz, 1H, OCHHCHN), 2.96 (m, 2H, CH_2CH_3), 2.40 (m, 1H, $CH(CH_3)_2$), 1.18 (t, 7.5 Hz, 3H, CH_2CH_3), 0.94 (d, 7.1 Hz, 3H, $CH(CH_3)_2$), 0.88 (d, 6.9 Hz, 3H, $CH(CH_3)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4, 153.7, 63.0, 57.9, 28.5, 28.1, 17.3, 14.2, 7.9; exact mass 185.1051 (185.1052 calculated for $C_9H_{15}NO_3$).

General Procedure for the Cleavage of Aminated Carboximides with Lithium Alkoxide ($LiOCH_2Ph$)

(*S*)-Benzyl 2-[1,2-bis(benzyloxycarbonyl)hydrazino]-3-methylbuyrate (57)

The removal of the oxazolidinone **29** was carried out using literature procedures.[99] To a cooled solution (0 °C) of **53** (298 mg, 0.58 mmol) in THF (5 mL) was added a solution of lithium benzylalkoxide in THF (2.5 mL, 0.47 M, 0.120 mmol). The mixture was stirred for 8 min and then quenched by the addition of aqueous 5% NH_4Cl (10 mL). The mixture was then extracted with dichloromethane (2 x 20 mL), and the combined

organic phases were dried and concentrated *in vacuo* to give a yellow oil (452 mg). The crude product was purified by flash chromatography[246] (30% ethyl acetate/70%hexane) to afford 65.9 mg (88% yield) of the chiral auxillary **29** and 246 mg of **57** (86% yield) as a white solid: mp 71 - 73 °C; IR ($CHCl_3$ cast) 3312 br, 1781, 1740, 1722, 1389, 1302, 1216 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.24 (br s, 16H, Ph + NH), 5.17 (s, 2H, $CHCOOCH_2Ph$), 5.09 (s, 4H, $NCOOCH_2Ph$), 4.61 (br d, 8.0 Hz, 1H, NCHCO), 2.25 (m, 1H, $CH(CH_3)_2$), 0.95 (br d, 6.4 Hz, 6H, $CH(CH_3)_2$); MS 508 (CI, M + NH_4^+); Anal. Calc. for $C_{28}H_{30}N_2O_6$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.45; H, 6.16; N, 5.75. Hydrogenolysis to **60** (see below) followed by formation of the camphanamide methyl ester[125] **86** and GC diastereomer analysis (DB-17+, 150 °C for 2 min, 2 °C/min to 220 °C, 220 °C for 5 min, 9.5 psi) afforded a ratio of 2.9 : 97.1 for the 2*R* (t_r = 22.67 min) and 2*S* (t_r = 24.25 min) isomers.

General Procedure for the Generation of α-Hydrazinocarboxylic Acids from Their Protected Forms and the Determination of Their Enantiomeric Purity

(*S*) 2-Hydrazino-3-methylbutyric acid hydrochloride (60)

A suspension of **57** (502 mg, 1.0 mmol), 5% Pd/C (10 mg), and 1.5 mL 6N HCl in THF (8 mL) was stirred under an atmosphere of H_2 for 16 h. The catalyst was removed by filtration and washed with 0.5N HCl (3 x 10 mL). The combined aqueous phases were taken to dryness *in vacuo* to afford a pale brown solid. A portion of this solid was retained for conversion to the (-)-camphanamide methyl ester[125] and the remainder was recrystallized from ethanol/water to give 169 mg (98% yield) of **60** as its hydrochloride salt: $[\alpha]_D$ = -10.8° (c 1.8, 6N HCl), lit.[89] $[\alpha]_D$ = -11° (c 1.0, 6N HCl); mp 155 - 158 °C dec; IR (KBr disk) 3238, 2971 br, 1713, 1426, 1223, 856, 809, 709, 676 cm^{-1}; ^1H NMR (80 MHz, D_2O) δ 3.58 (d, 4.6 Hz, 1H, COCH), 2.13, (m, 1H, $CHCH(CH_3)_2$), 0.92 (d, 5.7 Hz, 3H, $CH(CH_3)_2$), 0.84 (d, 5.7 Hz, 3H, $CH(CH_3)_2$); MS 133 (Glycerol, FAB

MS, MH^+). GC diastereomer analysis of the camphanamide methyl ester[125] **86** afforded a ratio of 2.9 : 97.1 for the 2*R* (t_r = 22.67 min) and 2*S* (t_r = 24.25 min) isomers.

(*S*)-Benzyl 2-[1,2-bis(benzyloxycarbonyl)hydrazino]propionate (58)

The procedure used to transform **53** to **57** was employed to produce **58** from 2.50 g (5.2 mmol) of aminated carboximide **54**. The crude product was purified by flash chromatography[246] (33% ethyl acetate/67%hexane) to afford 2.28 g of **58** (93% yield) as a white solid: mp 76 - 78 °C; IR ($CHCl_3$ cast) 3318 br, 1738, 1722, 1301, 1215, 1070, 740 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.32 (br s, 15H, Ph) 6.84 (br s, 1H, NH), 5.13 (s, 6H, CH_2Ph), 5.08 (m, 1H, $CHCH_3$), 1.48 (d, 7.5 Hz, 3H, $CHCH_3$); exact mass 462.1780 (462.1791 calculated for $C_{26}H_{26}N_2O_6$). Anal. Calc. for $C_{26}H_{26}N_2O_6$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.18; H, 5.63; N, 6.04. Hydrogenolysis to **61** followed by enantiomer analysis by optical rotation afforded a ratio of 72 : 28 for the 2*S* and 2*R* isomers respectively.

(*S*)-Benzyl 2-[1,2-bis(benzyloxycarbonyl)hydrazino]-3-phenylpropionate (59)

The procedure used to transform **53** to **57** was employed to produce **59** from 218 mg (0.39 mmol) of aminated carboximide **55**. The crude product (301 mg) was purified by MPLC (50 g Merck Kieselgel 60H, 35% ethyl acetate/65% hexane, 3 mL/min) to afford 41 mg of the chiral auxillary **29** (83%) and 183 mg of **59** (87% yield) as a white solid: mp 85 - 86 °C; IR ($CHCl_3$ cast) 3300 br, 1737, 1721, 1497, 1405, 1215, 741 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.25 (m, 20H, Ph), 6.88 (br s, 1H, NH), 5.05 (br s, 7H, OCH_2Ph + $CHCH_2$), 3.22 (d, 6.6 Hz, 2H, $CHCH_2Ph$); exact mass 538.2106 (538.2104 calculated for $C_{32}H_{30}N_2O_6$). Anal. Calc. for $C_{32}H_{30}N_2O_6$: C, 71.36; H, 5.61; N, 5.20. Found: C, 71.25; H, 5.72; N, 5.28. Hydrogenolysis (see below) to **62** followed by formation of the camphanamide methyl ester[125] **83** and GC diastereomer analysis (DB-17+, 170 °C 2

min, 2 °C/min to 250 °C, 250 °C 5 min, 9.5 psi) afforded a ratio of 11.6 : 88.4 for the isomers 2*R* (t_r = 34.85 min) and 2*S* (t_r = 35.59 min) isomers.

(*S*) 2-Hydrazinopropionic acid (61) hydrochloride from 58

The procedure used to convert **57** to **60** was employed. Thus 703 mg of **58** (1.5 mmol) afforded 141 mg (92% yield) of **61** as its hydrochloride salt: $[\alpha]_D$ = -13.3° (c 1.0, 6N HCl), lit.[91] $[\alpha]_D$ = -30.3° (c 1.0, 6N HCl); mp 141 - 143 °C dec; IR (KBr disk) 3424 br, 1724 1402 1244, 1109 cm^{-1}; ^1H NMR (80 MHz, D_2O) δ 3.57 (q, 7.1 Hz, 1H, $CHCH_3$), 1.27 (d, 7.1 Hz, 3H, $CHCH_3$); MS 105 (Glycerol, FAB MS, MH^+).

(*S*) 2-Hydrazino-3-phenylpropionic acid (62) hydrochloride from 59

The procedure used to convert **57** to **60** was employed. Thus 112 mg of **59** (0.23 mmol) afforded 47.8 mg of a pale brown solid. A portion of this solid was retained for conversion to the (-)-camphanamide methyl ester[125] **83** and the remainder was recrystallized from ethanol/water to give 41.6 mg of **62** (83% yield): $[\alpha]_D$ = -12.1° (c 0.8, 6N HCl), lit.[89] $[\alpha]_D$ = -16° (c 0.7, 6N HCl); mp 187 - 189 °C dec; IR (KBr disk) 2940 br, 1729, 1495, 1230, 697 cm^{-1}; ^1H NMR (80 MHz, D_2O) δ 7.28 (br s, 5H, Ph), 4.11 (m, 1H, $CHCH_2$), 3.08 (d, 7.2 Hz, 2H, $CHCH_2$); MS 181 (Glycerol, FAB MS, MH^+). GC diastereomer analysis (DB-17+, 170 °C for 1 min, 2 °C/min to 240 °C, 9 psi) of the derived camphanamide methyl ester[125] **83** (see below) afforded a ratio of 11.9 : 88.1 for the 2*R* (t_r = 30.18 min) and 2*S* (t_r = 31.11 min) isomers.

***tert*-Butyl hydrazodiformate[124]**

A modification of the procedure by Paleveda *et al*[123] was employed. To a solution of hydrazine hydrate (98%, 5 g, 0.10 mmol) and 42 mL of triethylamine (0.30 mmol) in 200 mL of dioxane/water (1:1) was added, with stirring, 54.2 g (0.22 mmol) of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, Aldrich). The resulting yellow

slurry was stirred at room temperature overnight and then extracted with chloroform (3 x 100 mL). The combined organic phases were dried and concentrated *in vacuo* to give a yellow amorphous solid. The solid was recrystallized (1:1 benzene:pet. ether) to give 12.3 g (53% yield) of *tert*-butyl hydrazodiformate as white needles: mp 123 - 124 °C, lit.[124] mp 124 - 125.5 °C; IR ($CHCl_3$ cast) 3314, 1800, 1480, 1246, 1155 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 6.32 (br s, 2H, NH), 1.46 (s, 18H, CH_3).

tert-Butyl azodiformate (63)[124]

This compound was prepared as described in the literature[124] from *tert*-butyl hydrazodiformate: mp 88 - 91 °C , lit. mp[124] 90 - 91.5 °C; IR ($CHCl_3$ cast) 1767, 1369, 1279, 1259, 1142, 833 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) d 1.61 (s).

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(*tert*-butoxycarbonyl)-hydrazino]propyl)-4-isopropyloxazolidin-2-one (64)

The procedure used to transform **56** to **54** was employed to produce **64** from 185 mg (1.0 mmol) of carboximide **56** and *tert*-butyl azodiformate (**63**). The crude product was purified by flash chromatography[246] (ethyl acetate 30%/hexane 70%) to give 383 mg of **64** (92% yield) as an oil: IR ($CHCl_3$ cast) 3366 br, 2973, 1784, 1702, 1391, 1243, 755 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 6.63 (br s, 1H, NH), 5.73 (br q, 6.8 Hz, 1H, $CHCH_3$), 3.97 (m, 1H, OCHHCHN), 3.67 (dd, 9.6, 3.2 Hz, 1H, OCHHCHN), 3.58 (dd, 9.5, 9.5 Hz, 1H, OCHHCHN), 2.24 (m, 1H, $CH(CH_3)_2$), 1.51 (d, 7.0 Hz, 3H, $CHCH_3$), 1.44 (s, 9H, $C(CH_3)_3$),1.41 (s, 9H, $C(CH_3)_3$), 0.75 (d, 7.2 Hz, 3H, $CH(CH_3)_2$), 0.62 (d, 7.8 Hz, 3H, $CH(CH_3)_2$); ^{13}C NMR (100 MHz, toluene-d_8, 374 °K) δ 174.6, 155.7, 155.5, 153.4, 81.5, 80.4, 63.9, 59.3, 57.3, 29.1, 28.5, 28.4, 17.6, 15.1, 14.3; exact mass 415.2317 (415.2318 calculated for $C_{19}H_{33}N_3O_7$). Anal. Calc. for $C_{19}H_{33}N_3O_7$: C, 54.93; H, 8.01; N, 10.11. Found: C, 54.61; H, 7.85; N, 9.93.

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(*tert*-butoxycarbonyl)-hydrazino]-3-phenylpropyl)-4-isopropyloxazolidin-2-one (65)

The procedure used to transform **56** to **54** was employed to produce **65** from 392 mg (1.5 mmol) of carboximide **32** and di-*tert*-butyl azodiformate (**63**). The crude product was purified by flash chromatography[246] (ethyl acetate 30%/hexane 70%) to give 571 mg of **65** (78% yield) as a yellow oil: HPLC diastereomer analysis (48% hexane/48% dichloromethane/4% acetonitrile, 2 mL/min) afforded a ratio of 93:7 for the 3(2*S*) (t_r = 12.6 min) and 3(2*R*) (t_r = 16.0 min) isomers; IR ($CHCl_3$ cast) 3340 br, 2975, 1783, 1705, 1389, 1367, 1246 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.10 (m, 5H, Ph), 6.56 (br s, 1H, NH), 6.43 (t, 9.2 Hz, 1H, $CHCH_2Ph$), 3.87 (m, 1H, OCHHCHN), 3.56 (dd, 8.8, 4.8 Hz, 1H, OCHHCHN), 3.35 (m, 3H, OCHHCHN + CH_2Ph), 2.22 (m, 1H, under toluene signal, $CH(CH_3)_2$), 1.42 (s, 18H, $C(CH_3)_3$), 0.73 (d, 8.9 Hz, 3H, $CH(CH_3)_2$), 0.59 (d, 8.9 Hz, 3H, $CH(CH_3)_2$); exact mass 491.2613 (491.2631 calculated for $C_{25}H_{37}N_3O_7$). Anal. Calc. for $C_{25}H_{37}N_3O_7$: C, 61.08; H, 7.59; N, 8.55. Found: C, 61.37; H, 7.59; N, 8.40.

(*S*)-Benzyl 2-[1,2-bis(*tert*-butoxycarbonyl)hydrazino]propionate (66)

The procedure used to transform **53** to **57** was employed to produce **66** from 275 mg (0.70 mmol) of aminated carboximide **64**. The crude product was purified by flash chromatography[246] (20% ethyl acetate/80%hexane) to afford 199 mg of **66** (74% yield) as an oil: IR ($CHCl_3$ cast) 3325 br, 2978, 1748, 1742, 1712, 1368, 753 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.34 (br s, 5H, Ph) 6.39 (br s, 1H, NH), 5.15 (s, 2H, CH_2Ph), 4.91 (br m, 1H, $CHCH_3$), 1.45 (m, 21H, $CHCH_3$ + $C(CH_3)_3$); exact mass 321.1454 (321.1450 calculated for $C_{16}H_{21}N_2O_5$, $M^+ - C_4H_9O$); MS 412 (CI, M + NH_4^+). Anal. Calc. for $C_{20}H_{30}N_2O_6$: C, 60.90; H, 7.67; N, 7.06. Found: C, 60.93; H, 7.68; N, 7.08. Hydrolytic cleavage by sequential treatment with trifluoroacetic acid (5 mL, 30 min)

and 6N HCl (5 mL, reflux, 2 h) to **61** followed by enantiomer analysis by optical rotation afforded a ratio of 86 : 14 for the 2*S* and 2*R* isomers respectively.

(*S*)-Benzyl 2-[1,2-bis(*tert*-butoxycarbonyl)hydrazino]-3-phenylpropionate (67)

The procedure used to transform **53** to **57** was employed to produce **67** from 181 mg (0.37 mmol) of aminated carboximide **65**. The crude product was purified by flash chromatography[246] (25% ethyl acetate/75%hexane) to afford 142 mg of **67** (97% yield) as an oil: IR ($CHCl_3$ cast) 3320 br, 2979, 1739, 1717, 1366, 1254, 1242, 1154 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.25 (m, 10H, Ph) 6.37 (br s, 1H, NH), 5.12 (br s, 2H, OCH_2Ph), 4.08 (t, 7.1 Hz, 1H, $CHCH_2Ph$), 3.20 (d, 7.0 Hz, 2H, $CHCH_2Ph$), 2.45 (s, 18H, $C(CH_3)_3$); exact mass 470.2389 (470.2417 calculated for $C_{26}H_{34}N_2O_6$).

Hydrolytic cleavage by sequential treatment with trifluoroacetic acid (5 mL, 30 min) and 6N HCl (5 mL, reflux, 2 h) to **62** followed by enantiomer analysis by optical rotation afforded a ratio of 83 : 17 for the 2*S* and 2*R* isomers respectively.

(*S*) 2-Hydrazinopropionic acid (61) hydrochloride from 66

The procedure used to convert **67** to **62** was employed (see below). Thus 142 mg of **66** (0.36 mmol) afforded 36.1 mg (71% yield) of **61** as its hydrochloride salt: $[\alpha]_D$ = -22.0° (c 1.0, 6N HCl), lit.[91] $[\alpha]_D$ = -30.3° (c 1.0, 6N HCl). The chromatographic and spectral properties were identical to those of **61** prepared from **58**.

(*S*) 2-Hydrazino-3-phenylpropionic acid (62) hydrochloride from 67

Compound **67** (117 mg, 0.25 mmol) in trifluoroacetic acid (5 mL) was stirred for 30 min at 20 °C. The solvent was removed *in vacuo* and the residue was dissolved in 6N HCl and heated to reflux for 2 h. The reaction mixture was cooled, washed with diethyl ether (2 x 10 mL), and concentrated *in vacuo* to give 43.8 mg (81% yield) of **62** as its

hydrochloride salt: $[\alpha]_D$ = -10.7° (c 1.1, 6N HCl), lit.[89] $[\alpha]_D$ = -16° (c 0.7, 6N HCl); all other physical constants were identical to those of **62** obtained from **59**.

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(methoxycarbonyl)-hydrazino]-3-phenylpropyl)-4-isopropyloxazolidin-2-one (68)

The procedure used to transform **56** to **54** was employed to produce **68** from 46.0 mg (0.18 mmol) of carboximide **32** and dimethyl azodiformate. The crude product was purified by flash chromatography[246] (ethyl acetate 40%/hexane 60%) to give 60.8 mg of **68** (83% yield) as a white solid: HPLC diastereomer analysis (45% hexane/45% dichloromethane/10% acetonitrile, 1 mL/min) afforded a ratio of 69 : 31 for the 3(2*S*) (t_r = 39.7 min) and 3(2*R*) (t_r = 37.0 min) isomers; mp 153 - 154 °C; IR ($CHCl_3$ cast) 3322 br, 1780, 1720, 1388, 1373, 1301 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.32 (m, 5H, Ph), 7.13 (br s, 1H, NH), 6.31 (br t, 7.6 Hz, 1H, $CHCH_2Ph$), 3.86 (m, 1H, $OCHHCHN$), 3.54 (dd, 8.8, 5.5 Hz, 1H, $OCHHCHN$), 3.42 (s, 6H, OCH_3), 3.32 (m, 3H, $OCHHCHN$ + CH_2Ph), 2.07 (m, 1H, under toluene signal, $CH(CH_3)_2$), 0.67 (d, 9.6 Hz, 3H, $CH(CH_3)_2$), 0.56 (d, 9.6 Hz, 3H, $CH(CH_3)_2$); exact mass 376.1500 (376.1508 calculated for $C_{18}H_{22}N_3O_6$, M^+ - MeO); MS 408 (CI, MH^+). Anal. Calc. for $C_{19}H_{25}N_3O_7$: C, 56.01; H, 6.18; N, 10.31. Found: C, 55.71; H, 6.20; N, 9.97.

[3(2*S*),4*S*]-3-(1-Oxo-2-[1,2-bis(ethoxycarbonyl)-hydrazino]-3-phenylpropyl)-4-isopropyloxazolidin-2-one (69)

The procedure used to transform **56** to **54** was employed to produce **69** from 37.4 mg (0.14 mmol) of carboximide **32** and diethyl azodiformate. The crude product was purified by flash chromatography[246] (ethyl acetate 35%/hexane 65%) to give 53.7 mg of **69** (88% yield) as a white solid: HPLC diastereomer analysis (46% hexane/46% dichloromethane/8% acetonitrile, 1 mL/min) afforded a ratio of 75 : 25 for the 3(2*S*) (t_r = 20.8 min) and 3(2*R*) (t_r = 25.2 min) isomers; mp 91 - 93 °C; IR ($CHCl_3$ cast) 3308 br,

1782, 1715, 1387, 1301, 1204 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.30 (m, 5H, Ph), 7.05 (br s, 1H, NH), 6.30 (br t, 7.6 Hz, 1H, $CHCH_2Ph$), 4.05 (m, 4H, CH_2CH_3) 3.86 (m, 1H, OCHHCHN), 3.55 (dd, 8.9, 5.0 Hz, 1H, OCHHCHN), 3.34 (m, 3H, OCHHCHN + CH_2Ph), 2.20 (m, 1H, under toluene signal, $CH(CH_3)_2$), 1.02 (m, 6H, CH_2CH_3), 0.68 (d, 9.5 Hz, 3H, $CH(CH_3)_2$), 0.56 (d, 9.6 Hz, 3H, $CH(CH_3)_2$); exact mass 435.1978 (435.2005 calculated for $C_{21}H_{29}N_3O_7$). Anal. Calc. for $C_{21}H_{29}N_3O_7$: C, 57.92; H, 6.71; N, 9.65. Found: C, 57.53; H, 6.72; N, 9.71.

[3(2*R*),4*R*]-3-(1-Oxo-2-[1,2-bis(benzyloxycarbonyl)-hydrazino]propyl)-4-isopropyloxazolidin-2-one (70)

The procedure used to transform **56** to **54** was employed to produce **70** from 1.50 g (8.1 mmol) of carboximide **71** and dibenzyl azodiformate. The crude product was purified by flash chromatography[246] (32% ethyl acetate/68% hexane) to afford 3.11 g of **70** (79% yield) as a white solid: HPLC diastereomer analysis (46% hexane/46% dichloromethane/8% acetonitrile, 1 mL/min) afforded a ratio of 90 : 10 for the 3(2*R*) (t_r = 14.6 min) and 3(2*S*) (t_r = 19.0 min) isomers; mp 117 - 119 °C. MS, IR, and NMR spectral data were identical to the [3(2S), 4S] enantiomer **54**.

(*R*)-3-(1-Oxopropyl)-4-isopropyloxazolidin-2-one (71)

This material was prepared as described for **56** by using propionyl chloride (1.44 g, 16 mmol) and the oxazolidinone (**30**) (2.00 g, 16 mmol) to produce 2.43 g (85% yield) of **71** as a colourless oil. ^1H NMR, IR, and MS spectra of **71** were identical to those of **56**.

[3(2*R*),4*S*]-3-(1-Oxo-2-[1,2-bis(benzyloxycarbonyl)-hydrazino]propyl)-4-isopropyloxazolidin-2-one (72)

HPLC purification (46% hexane/46%dichloromethane/8%acetonitrile, 1 mL/min) by repeated injection of a 90 : 10 mixture of the 3(2*S*) and 3(2*R*) isomers gave a small sample of **72** (0.23 mg). Reinjection of **72** on the HPLC gave a peak that did not elute with the major (3(2*S*) diastereomer: IR ($CHCl_3$ cast) 3320 br, 1782, 1754, 1720 br, 1388, 1218, 1207, 796 cm^{-1}; exact mass 483.2006 (483.2005 calculated for $C_{25}H_{29}N_3O_7$).

[3(2*R*),4*S*]-3-(1-Oxo-2-[1,2-bis(benzyloxycarbonyl)-hydrazino]-3-phenylpropyl)-4-isopropyloxazolidin-2-one (73)

MPLC purification (ethyl acetate 35%/ hexane 65%, 55 g silica gel) of a 94 : 6 mixture (460 mg) of the 3(2*S*) and 3(2*R*) isomers obtained by the reaction of carboximide **32** with dibenzyl azodiformate produced one major peak with a shoulder on its leading front. The fractions that corresponded to this shoulder were combined and concentrated *in vacuo* to give 3.11 mg of the 3(2*R*) material as a colourless oil. Reinjection of **73** on the HPLC gave a peak that did not elute with the major (3(2*S*) diastereomer: IR ($CHCl_3$ cast) 3305 br, 2960, 1784, 1720,1708, 1389, 1217, 750, 698 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.40 (m, 15H, Ph), 6.68 (br s, 1H, NH), 6.55 (dd, 8.5, 6.4 Hz, 1H, CHCH$_2$Ph), 4.96 (m, 4H, OCH$_2$Ph), 3.90 (dt, 5.8, 5.3 Hz, 1H, OCHHCHN), 3.50 (br d, 5.2 Hz, 2H, OCHHCHN), 3.30 (dd, 14.0, 6.4 Hz, 1H, COCHCHHPh), 3.19 (dd, 13.8, 8.2 Hz, 1H, COCHCHHPh), 1.96 (m, 1H, CH(CH$_3$)$_2$), 0.51 (d, 5.7 Hz, 3H, CH(CH$_3$)$_2$), 0.49 (d, 4.9 Hz, 3H, CH(CH$_3$)$_2$); exact mass 559.2315 (559.2318 calculated for $C_{31}H_{33}N_3O_7$).

Preparation of GC Standards of Camphanamide Methyl Esters

(*S*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]amino)-3-phenylpropionate (76)[126]

Modified literature procedures[125] were employed. To a stirred solution of L-phenylalanine (191 mg, 1.16 mmol) in 2 mL aqueous KOH (2N) was added a solution of the (-)-camphanoyl chloride (377 mg, 1.73 mmol) in toluene (2 mL). The pH was maintained at 10 by the occasional addition of 2N KOH. The aqueous phase was diluted after 2 h with 30 mL of water, washed with dichloromethane (30 mL), acidified to pH 1 with 6N HCl, and extracted with dichloromethane (3 x 15 mL). The combined organic phases were then dried, concentrated *in vacuo*, and redissolved in dichloromethane (5 mL). A solution of diazomethane in dichloromethane was then added dropwise until the yellow colour remained. After removal of the solvent *in vacuo* the resulting oil was purified by MPLC (ethyl acetate 35%/hexane 65%, 50 g silica gel, 3 mL/min) to give 238 mg of **76** (57% yield) as a colourless oil: IR ($CHCl_3$ cast) 3362 br, 1790, 1742, 1647, 1542, 921 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.21 (m, 5H, Ph), 6.92 (br d, 9.2 Hz, 1H, NH), 4.94 (m, 1H, $CHCH_2Ph$), 3.70 (s, 3H, OMe), 3.24 (dd, 13.6, 5.6 Hz, 1H, $CHCHHPh$), 3.04 (dd, 13.6, 8.7 Hz, 1H, $CHCHHPh$), 2.48 (m, 1H, (-)-camphanamide), 1.89 (m, 2H, (-)-camphanamide), 1.66 (m, 1H, (-)-camphanamide), 1.06 (s, 3H, Me), 1.00 (s, 3H, Me), 0.60 (s, 3H, Me); exact mass 359.1733 (359.1732 calculated for $C_{20}H_{25}NO_5$). Anal. Calc. for $C_{20}H_{25}NO_5$: C, 66.83; H, 7.01; N, 3.90. Found: C, 66.66; H, 7.05; N, 3.68.

Mixture of (*S*) and (*R*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]amino)-3-phenylpropionate (77)[126]

The procedure used to prepare **76** from L-phenylalanine was employed. Thus, a mixture of L-phenylalanine (31.7 mg, 0.19 mmol) and D,L-phenylalanine (32.4 mg, 0.20 mmol) was treated with (-)-camphanoyl chloride (126 mg, 0.58 mmol) and methylated as

previously described. However, the mixture was not purified by MPLC but instead submitted directly to GC analysis (DB-17+, 170 °C for 1 min, 2 °C/min to 240 °C, 9 psi) to afford a ratio of 27.2 : 72.8 for the (*R*) (t_r = 29.19 min) and (*S*) (t_r = 29.99 min) compounds respectively (calculated ratio 25.3 : 74.7).

(*S*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]amino)propionate (79)

The procedure used to produce **76** was employed to produce **79** (559 mg, 72%) as a crystalline solid from 211 mg of L-alanine (2.73 mmol) and 771 mg of (-)-camphanoyl chloride (3.56 mmol): mp 69 - 69.5 °C; IR ($CHCl_3$ cast) 3368 br, 1792, 1746, 1675, 1528, 1190, 1063 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.14 (br d, 7.3 Hz, 1H, NH), 4.66 (p, 7.2 Hz, 1H, $CHCH_3$), 3.79 (s, 3H, OMe), 2.50 (m, 1H, (-)-camphanamide), 1.95 (m, 3H, (-)-camphanamide), 1.45 (d, 7.3 Hz, $CHCH_3$), 1.15 (s, 6H, Me), 0.93 (s, 3H, Me); exact mass 283.1423 (283.1419 calculated for $C_{14}H_{21}NO_5$). Anal. Calc. for $C_{14}H_{21}NO_5$: C, 59.35; H, 7.47; N, 4.97. Found: C, 59.28; H, 7.41; N, 5.02.

Mixture of (*S*) and (*R*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]amino)propionate (81)

The mixture **81** was prepared from L-alanine (59.6 mg, 0.67 mmol) and D,L-alanine (40.1 mg, 0.45 mmol) as previously described for mixture **77**. GC analysis (DB-17+, 50 °C 1 min, 25 °C/min to 100 °C, 100 ° C for 1 min, 2 °C/min to 180 °C, 180 °C for 5 min, 9.5 psi), afforded a ratio of 18.2 : 81.8 for the (*R*) (t_r = 41.05 min) and (*S*) (t_r = 41.56 min) compounds, respectively (calculated ratio 20.0 : 80.0).

(*S*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]amino)-3-methylbutyrate (80)

L-valine (156 mg, 1.33 mmol) was converted to **80** (398 mg, 96% yield) as previously described to give a colourless oil: IR ($CHCl_3$ cast) 3370 br, 1795, 1743, 1678, 1523, 1187 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.89 (br d, 8.9 Hz, 1H, NH), 4.58 (dd, 9.0, 5.2 Hz, 1H, COCH), 3.76 (s, 3H, OMe), 2.49 (m, 1H, (-)-camphanamide), 1.98 (m, 3H, (-)-camphanamide), 1.71 (m, 1H, $CH(CH_3)_2$), 1.23 (s, 3H, Me), 1.12 (s, 3H, Me), 0.96 (d, 7.0 Hz, 3H, $CH(CH_3)_2$), 0.94 (s, 3H, Me), 0.92 (d, 7.0 Hz, 3H, $CH(CH_3)_2$); exact mass 311.1727(311.1732 calculated for $C_{16}H_{25}NO_5$). Anal. Calc. for $C_{16}H_{25}NO_5$: C, 61.72; H, 8.09; N, 4.50. Found: C, 61.35; H, 8.16; N, 4.31.

Mixture of (*S*) and (*R*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]amino)-3-methylbutyrate (82)

The mixture **82** was prepared from L-valine (46.0 mg, 0.39 mmol) and D,L-valine (19.7 mg, 0.17 mmol) as previously described for mixture **77**. GC analysis (DB-17+, 80 °C for 1 min, 5 °C/min to 220 °C , 25 °C/min to 250 °C, 250 °C for 5 min, 9.5 psi), afforded a ratio of 13.5 : 86.5 for the (*R*) (t_r = 24.91 min) and (*S*) (t_r = 25.51 min) compounds, respectively (calculated ratio 14.8 : 85.2).

Preparation of a Mixture of (*S*) and (*R*) Methyl 2-(2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]hydrazino)-3-phenylpropionate (83) and a mixture of (*S*) and (*R*) Methyl 2-(1,2-bis[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]hydrazino)-3-phenylpropionate (84)

Modified literature procedures[125] were employed. To a solution of (-)-camphanoyl chloride (166 mg, 0.76 mmol) in toluene (0.2 mL) was added a solution of the hydrazinocarboxylic acid hydrochloride salt **62** (110 mg, 0.47 mmol, (*S*) : (*R*) ratio 70 :

30) in 0.8 mL 2N KOH. The mixture was stirred for 2 h, washed with dichloromethane (2 x 2 mL), acidified to pH 1 with 6N HCl, and extracted with dichloromethane (3 x 5 mL). The combined organic phases were dried, concentrated *in vacuo* , dissolved in ether (3 mL). A solution of diazomethane in ether was added dropwise until the yellow colour remained. The solvent was removed *in vacuo* to give a golden oil that was purified by column chromatography (50 g silica gel, ethyl acetate gradient (10% steps, 100 mL each, 10% to 50%) in hexane) to give a 7 : 3 mixture of diastereomers **83a** and **83b** (110 mg, 63% yield) as a colourless oil and a 7 : 3 mixture of diastereomers **84a** and **84b** (54.7 mg, 21% yield) as a colourless oil. For **83a** and **83b**: IR ($CHCl_3$ cast) 3288 br, 2960, 1792, 1741, 1667, 1452, 1165, 921 cm^{-1}; ^1H NMR of **83a** (400 MHz, $CDCl_3$) δ 7.92 (br s, 1H, NHNHCO), 7.30 (m, 5H, Ph), 4.97 (br t, 6.0 Hz, 1H, NHNHCO), 3.87 (m, 1H, $CHCH_2Ph$), 3.76 (s, 3H, OMe), 3.14 (dd, 13, 5.6 Hz, 1H, $CHCHHPh$), 2.98 (m, 1H, $CHCHHPh$), 2.74 (m, 1H, (-)-camphanamide), 1.88 (m, 2H, (-)-camphanamide), 1.65 (m, 1H, (-)-camphanamide), 1.10 (s, 3H, Me), 1.07 (s, 3H, Me), 0.86 (s, 3H, Me); ^1H NMR of **83b** (400 MHz, $CDCl_3$) δ 7.91 (br s, 1H, NHNHCO), 7.30 (m, 5H, Ph), 4.83 (br t, 5.0 Hz, 1H, NHNHCO), 3.98 (m, 1H, $CHCH_2Ph$), 3.72 (s, 3H, OMe), 3.14 (dd, 13, 5.6 Hz, 1H, $CHCHHPh$), 2.98 (m, 1H, $CHCHHPh$), 2.47 (m, 1H, (-)-camphanamide), 1.88 (m, 2H, (-)-camphanamide), 1.65 (m, 1H, (-)-camphanamide), 1.10 (s, 3H, Me), 1.08 (s, 3H, Me), 0.87 (s, 3H, Me); exact mass 374.1833 (374.1841 calculated for $C_{20}H_{26}N_2O_5$). Anal. Calc. for $C_{20}H_{26}N_2O_5$: C, 64.16; H, 7.00; N, 7.48. Found: C, 63.84; H, 6.90; N, 7.08. For **84a** and **84b**: IR ($CHCl_3$ cast) 3318 br, 2967, 1793, 1746, 1718, 1675, 1268, 1168, 926 cm^{-1}; ^1H NMR for **84a** (400 MHz, $CDCl_3$) δ 8.28 (br s, 0.5H, NH), 8.21 (br s, 0.5H, NH), 7.20 - 7.05 (m, 5H, Ph), 5.47 (t, 7.6 Hz, 0.5H, $CHCHHPh$), 5.10 (t, 7.3 Hz, 0.5H, $CHCHHPh$), 3.80 - 3.65 (m, 3H, OMe), 3.28 - 3.02 (m, 2H, $CHCHHPh$), 2.60 - 1.50 (m, 4H, (-)-camphanamide), 1.27 - 0.51 (m, 18H, Me); ^1H NMR for **84b** (400 MHz, $CDCl_3$) δ 8.15 (br s, 0.4H, NH), 8.07 (br s, 0.6H, NH), 4.99 (m, 1H, $CHCHHPh$), 3.80 - 3.65 (m, 3H, OMe), 3.28 - 3.02 (m,

2H, CHCHHPh), 2.60 - 1.50 (m, 4H, (-)-camphanamide), 1.27 - 0.51 (m, 18H, Me); exact mass 495.2526 (495.2495 calculated for $C_{28}H_{35}N_2O_6$, M^+ - COOMe); MS 572 (CI, M + NH_4^+). Anal. Calc. for $C_{30}H_{38}N_2O_8$: C, 64.97; H, 6.91; N, 5.05. Found: C, 64.97; H, 6.81; N, 4.72.

General Procedure for the Preparation of Camphanamide Methyl Esters From α-Hydrazinocarboxylic Acids

(*S*) Methyl 2-[(1*S*,4*R*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyl]hydrazino)-3-phenylpropionate (83)

A modification of literature procedures[125] was employed. To a stirred solution of unpurified **62** (obtained from hydrogenolysis of **59**) (11.2 mg, 0.047 mmol) in 1 mL pH 10 buffer (1M $NaHCO_3/Na_2CO_3$) was added a solution of (-)-camphanoyl chloride (28.9 mg, 0.13 mmol) in toluene (1 mL). The mixture was diluted after 2 h with water (5 mL) and washed with chloroform (2 x 10 mL). The aqueous phase was acidified to pH 1 with 6N HCl and extracted with chloroform (3 x 10 mL). The combined organic phases were dried, concentrated *in vacuo*, and dissolved in dichloromethane (2 mL). A solution of diazomethane in ether was added until the yellow colour remained. The residue obtained after removal of the solvent was redissolved in ethyl acetate (0.5 mL) and submitted for GC analysis. The GC diastereomer analysis (DB-17+, 170 °C for 2 min, 2 °C/min to 250 °, 250 °C for 5 min, 9.5 psi) afforded a ratio of 11.6 : 88.4 for the 2(*R*) (t_r = 34.85 min) and 2(*S*) (t_r = 35.59 min) isomers.

Epimerization Study of 55

Removal of the chiral auxillary from aminated carboximide **55** was carried out as described previously with the following exceptions: the reaction was done at room temperature and was worked up after 3h. Thus, after MPLC purification, 87.3 mg (91% yield) of **59** was obtained by cleavage of 100 mg (0.18 mmol) of carboximide **55** .

Hydrogenolysis to **62** followed by formation of the camphanamide methyl ester[125] **83** and GC analysis as before gave a ratio of 56.9 : 43.1 for the *S* and *R* isomers.

(*S*)-Methyl 2-[1,2-bis(benzyloxycarbonyl)hydrazino]propionate (87)

The procedure of Evans *et al*[97] was followed. To distilled methanol (3 mL) in a 25 mL round bottom flask under Ar was added isopropylmagnesium chloride (0.79 mmol, 2M), and the mixture was stirred for 5 min at 0 °C. A solution of 191 mg of **54** (0.39 mmol) in methanol (3 mL) was added and stirring was continued for 10 min. The reaction was quenched with 1N H_3PO_4 (2 mL) followed by water (3 mL), and extracted with dichloromethane (3 x 5 mL). The combined organic phases were dried and concentrated *in vacuo* to give 141 mg of **87** (93% yield) as a colourless oil after flash chromatography[246] (33% ethyl acetate/67% hexane): $[\alpha]_D$ = 0.0° (c 2.8, $CHCl_3$); IR ($CHCl_3$ cast) 3310 br, 1741, 1720, 1410, 1303, 1216, 1071, 695 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.25 (s, 10H, Ph), 6.96 (br s, 1H, NH), 5.13 (s, 4H, CH_2Ph), 4.97 (br m, 1H, $CHCH_3$), 3.61 (s, 3H, OCH_3), 1.45 (d, 8.0 Hz, $CHCH_3$); exact mass 386.1479 (386.1478 calculated for $C_{20}H_{22}N_2O_6$.

(*S*)-Methyl 2-[1,2-bis(benzyloxycarbonyl)hydrazino]-3-phenylpropionate (88)

The procedure used to transform **54** to **87** was employed to produce **88** from 71.1 mg (0.13 mmol) of aminated carboximide **55**. The crude product was purified by flash chromatography[246] (ethyl acetate 35%/hexane 65%) to give 43.4 mg (73% yield) of **88** as a colourless oil: $[\alpha]_D$ = +0.1° (c 2.0, $CHCl_3$); IR ($CHCl_3$ cast) 3300 br, 1739, 1725, 1293, 1275, 1216, 698 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.25 (br s, 15H, Ph), 6.22 (br s, 1H, NH), 5.09 (s, 5H, OCH_2Ph + $CHCH_2$), 3.61 (s, 3H, OCH_3); 3.20 (d, 6.6 Hz, 2H, $CHCH_2Ph$); exact mass 462.1810 (462.1791 calculated for $C_{26}H_{26}N_2O_6$).

(*S*)-Benzyl 2-[1,2-bis(benzyloxycarbonyl)hydrazino]-3-phenylthiopropionate (89)

To a cooled (0 °C) solution of aminated carboximide **55** (51.4 mg, 0.092 mmol) in THF (4 mL) was added to a solution of lithium benzylthiolate (1 mL, 0.1M) in THF. The reaction was quenched with 10% NH_4Cl (5 mL) after stirring for 3 min and extracted with dichloromethane (2 x 20 mL). The combined organic phases were dried over $MgSO_4$, and taken to dryness *in vacuo*. Purification of the crude product by flash chromatography[246] (ethyl acetate 33%/hexane 67%) gave 47.5 mg of **89** (93% yield) as a colourless oil that solidified on standing: $[\alpha]_D$ = -0.1° (c 0.71, $CHCl_3$); mp 93 - 94.5 °C; IR ($CHCl_3$ cast) 3300 br, 1723, 1692, 1496, 1454, 1401, 1218, 742 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.33 (m, 21H, Ph + NH), 6.66 (br m, 1H, $CHCH_2$), 5.20 (s, 4H, OCH_2Ph), 4.13 (s, 2H, SCH_2Ph), 3.27 (d, 7.0 Hz, 2H, $CHCH_2Ph$); exact mass 431.1600 (431.1607 calculated for $C_{25}H_{23}N_2O_5$, $M^+ - C_7H_7S$); MS 572 (CI, M + NH_4^+). Anal. Calc. for $C_{32}H_{30}N_2O_5S$: C, 69.29; H, 5.45; N, 5.05. Found: C, 69.07; H, 5.48; N, 4.94.

Preparation of a Stock Suspension of LiSH in THF

Into a cooled (0 °C) 250 mL round bottom flask containing THF (100 mL) was placed 50 mL of nBuLi (1.23 M in hexane, 61.5 mmol). Dry H_2S gas was then passed through the solution until H_2S consumption halted. The resulting white suspension could then be stored for several weeks in the refrigerator with negligible decomposition.

General Procedure for the Cleavage of Aminated Carboximides with Lithium Hydrosulfide

(*R*) 2-[1,2-bis(benzyloxycarbonyl)hydrazino]propionic acid (91)

A suspension of LiSH in THF (5 mL) was added over 8 min to a stirred solution of **70** (53.2 mg, 0.11 mmol) in THF (3 mL). The mixture was stirred for 5 min, quenched with aqueous 5% NH_4Cl (5 mL), and acidified to pH 1 with 6N HCl. The combined

organic phases were extracted with chloroform (3 x 20 mL), dried, and concentrated *in vacuo* to give a yellow oil. The oil was dissolved in THF (2 mL) and added to a solution of AcOOH (40% AcOOH in water, 10 mL) and THF (10 mL). The solution was stirred for 15 min, brine (50 mL) was added, and the mixture was extracted with chloroform (3 x 40 mL). The combined organic phases were dried and concentrated *in vacuo* to give 72.1 mg of a colourless oil. Purification of the oil by MPLC (Merck Lobar RP-8 Size A and B in series, 60% methanol/40% water 600 mL, 80% methanol/20% water 600 mL, 3 mL/min) afforded 31.18 mg (76%) of **91** as a colourless oil: IR ($CHCl_3$ cast) 3280 br, 1720 br, 1302, 1260, 1220, 1069, 695 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 7.06 (m, 10H, Ph), 6.05 (br s, 1H, NH), 5.89 (br s, 1H, COOH), 4.95 (m, 4H, OCH_2Ph), 4.68 (br q, 7.3 Hz, 1H, $CHCH_3$), 1.30 (d, 7.3 Hz, 3H, $CHCH_3$); exact mass 372.1303 (372.1321 calculated for $C_{19}H_{20}N_2O_6$). Anal. Calc. for $C_{19}H_{20}N_2O_6$: C, 61.28; H, 5.41; N, 7.52. Found: C, 61.49; H, 5.53; N, 7.52. Hydrogenolysis to **92** (see below) followed by formation of the camphanamide methyl ester[125] **85** and GC diastereomer analysis (DB-17+, 50 °C for 1 min, 25 °C/min to 150 °C, 1.5 °C/min to 220 °C, 220 °C for 10 min, 6.5 psi) afforded a ratio of 88 : 12 for the 2*S* (t_r = 52.1 min) and 2*R* (t_r = 52.7 min) isomers.

(*R*) 2-Hydrazinopropionic acid (92) hydrochloride from 91

A 98% yield of **92** (18.0 mg) as its hydrochloride salt was obtained from 39 mg of **91** (0.11 mmol) when the procedure used to transform **57** to **60** was employed: GC diastereomer analysis (DB-17+, 50 °C for 1 min, 25 °C/min to 150 °C, 150 °C 1 min, 1.5 °C/min to 220 °C, 220 °C for 10 min, 6.5 psi) of the camphanamide methyl ester[125] **85** afforded a ratio of 88 : 12 for the 2*R* (t_r = 52.1 min) and 2*S* (t_r = 52.7 min) isomers.

(*S*) 2-[1,2-bis(benzyloxycarbonyl)hydrazino]-3-phenylpropionic acid (93)

The procedure used to transform **70** to **91** was employed to convert aminated carboximide **55** (152 mg, 0.27 mmol) to **93** (103 mg, 85% yield) which was obtained as a colourless oil: IR ($CHCl_3$ cast) 3260 br, 1721 br, 1405, 1213, 740, 694 cm^{-1}; ^1H NMR (400 MHz, toluene-d_8, 374 °K) δ 8.31 (br s, 1H, COOH), 7.02 (m, 15H, Ph), 6.46 (br s, 1H, NH), 4.95 (s, 2H, OCH_2Ph), 4.93 (s, 2H, OCH_2Ph), 4.87 (br s, 1H, $COCHCH_2Ph$), 3.25 (dd, 17, 8.0 Hz, 1H, $CHCHHPh$), 3.13 (dd, 17, 10 Hz, 1H, $CHCHHPh$); exact mass 403.1663 (403.1658 calculated for $C_{24}H_{23}N_2O_4$ M^+ - COOH), MS 466 (CI, M + NH_4^+). Hydrogenolysis to **62** (see below) followed by formation of the camphanamide methyl ester[125] **83** and GC diastereomer analysis (DB-17+, 170 °C for 2 min, 2 °C/min to 250 °C, 250 °C for 5 min, 9.5 psi) afforded a ratio of 13.8 : 86.2 for the 2*R* (t_r = 34.86 min) and 2*S* (t_r = 35.63 min) isomers. Note that the starting material **55** in this case had a 3(2*R*) : 3(2*S*) ratio of 13 : 87.

(*S*) 2-Hydrazino-3-phenylpropionic acid (62) acetate from 91

A suspension of **91** (53.9 mg, 0.12 mmol) and 5% Pd/C (5.2 mg) in AcOH (10 mL) was stirred rapidly under an atmosphere of hydrogen gas for 12 h. The catalyst was removed by filtration and washed with 5 mL AcOH. The combined organic phases were concentrated *in vacuo* to afford 23.6 mg (82% yield) of a white solid. The free hydrazinocarboxylic acid was obtained by dessicating the sample at 0.005 mm Hg for 48 h. The free hydrazino acid showed spectral constants consistent with previously obtained samples. GC diastereomer analysis (DB-17+, 170 °C for 1 min, 2 °C/min to 240 °C, 9 psi) of the camphanamide methyl ester[125] **83** afforded a ratio of 5.9 : 94.1 for the 2*R* (t_r = 30.18 min) and 2*S* (t_r = 31.11 min) isomers.

General Procedure for the Conversion of α-Hydrazinocarboxylic Acids to Their Parent α-Amino Acids and the Determination of Their Enantiomeric Purity

L-Phenylalanine Hydrochloride (73)

A suspension of **62** (*R* :*S* ratio of 11.6 : 88.4) (25.2 mg, 0.12 mmol) and Raney-nickel[131] (~ 10 mg) in 10% acetic acid/water (10 mL) was shaken under an atmosphere of hydrogen gas (500 psi) for 10 h. The catalyst was removed by filtration and washed with 0.5N HCl (3 x 3 mL). The combined aqueous phases were concentrated *in vacuo* , redissolved in 1N HCl (10 mL) and taken to dryness *in vacuo* to yield 22.7 mg (97% yield) of **73**. This was identical in all respects to authentic phenylalanine hydrochloride. Compound **73** was then converted[125] to its known[126] camphanamide methyl ester **76** as described previously and analysed by GC. The camphanamide ester was compared to standards prepared from pure D,L and pure L-phenylalanine. GC diastereomer analysis (DB-17+, 170 °C for 1 min, 2 °C/min to 240 °C, 9 psi) afforded a ratio of 11.7 : 88.3 for the 2(*R*) (t_r = 30.18 min) and 2(*S*) (t_r = 31.11 min) isomers.

L-Valine Hydrochloride (27)

A suspension of **60** (48.6 mg, 0.29 mmol) and Raney-nickel (~ 10 mg) in 0.6N HCl (10 mL) was shaken under an atmosphere of hydrogen gas (400 psi) for 10 h. The catalyst was removed by filtration and washed with 0.6N HCl (5 mL). The combined aqueous phases were taken to dryness *in vacuo* to give a pale green solid. This solid was converted to the camphanamide methyl ester[125] **80** as previously described and analysed by GC. The camphanamide ester was found to co-chromatograph with the authentic standards prepared from pure L and pure D,L-valine. GC diastereomer analysis (DB-17+, 80 °C for 1 min, 5 °C/min to 220 °C, 25 °C/min to 250 °C, 250 °C for 5 min, 9.5 psi) afforded a ratio of 2.9 : 97.1 for the (*R*) (t_r = 24.91 min) and (*S*) (t_r = 25.51 min) isomers, respectively.

[1-^{18}O] 2-Hydroxy-3,5,5-trimethylcyclohex-2-ene-1-one (109)

Modified literature procedures were followed.[194] A solution of epoxide **110** (1.81 g, 11.8 mmol), concentrated H_2SO_4 (0.10 mL) and 50% oxygen-18 labelled water (1.9 mL) was heated to reflux for 3 h. The cooled mixture was extracted with ether (3 x 15 mL). The combined organic phases were dried and concentrated *in vacuo* to give 1.6 g of a yellow oil. This material was recrystallized three times from ether/petroleum ether to give **109** as a fine white solid: mp 87 - 89 °C, lit.[194] mp 87 °C; IR ($CHCl_3$ cast) 3410 br, 1668, 1640, 1613, 1397, 1181, 679, 626; ^{1}H NMR (80 MHz, $CDCl_3$) δ 6.25 (s, 1H, O$\underline{H}$), 2.34 (s, 2H, COC$\underline{H}_2$), 2.27 (s, 2H, C=C-C$\underline{H}_2$), 1.85 (s, 3H, COHCC$\underline{H}_3$), 1.03 (s, 6H, C(C$\underline{H}_3$)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.0 s, 143.2 s, 127.6 s, 49.4 t, 44.7 t, 33.4 s, 28.3 q, 2 x 17.0 q; exact mass 154.0990 (154.0994 calculated for $C_9H_{14}{}^{16}O_2$), 156.1039 (156.1061 calculated for $C_9H_{14}{}^{16}O_1{}^{18}O_1$).

[$^{18}O_2$] Potassium peroxide[195] (113)

This compound was prepared as described in the literature.[195] Into a 50 mL 3-neck flask fitted with a gas inlet tube, a 60 mL addition funnel, and a mercury manometer was placed a suspension of 1.34 g potassium *tert*-butoxide (12 mmol) and 5 mL of benzene. The cooled (0 °C) apparatus was evacuated and filled with $^{18}O_2$ gas at 1 atmosphere pressure. A solution of benzhydrol (2.20 g, 12 mmol) in 32 mL benzene was added dropwise over a period of 10 min and the ice bath was removed. $^{18}O_2$ gas was added to the system to maintain a pressure of 1 atmosphere. Upon completion of $^{18}O_2$ uptake the mixture was stirred for 3 more hours during which time a precipitate formed. The slurry was then centrifuged and the solvent removed. The solid was resuspended in 10 mL of benzene, centrifuged and collected. The solid was dried for 16 h *in vacuo* to give 658 mg of **113** was obtained (76% yield) as a white solid.

[2-^{18}O] 2-Hydroxy-3,5,5-trimethylcyclohex-2-ene-1-one (112)[194]

Modified literature procedures were followed.[194] To a solution of 3,5,5-trimethylcyclohex-2-ene-1-one (**111**) (500 mg, 3.62 mmol) in 2 mL of methanol at 0 °C was added a solution of 658 mg of **113** (9.12 mmol) in 2 mL of water. The resulting yellow mixture was stirred for 3 h, extracted with dichloromethane (2 x 30 mL), dried and concentrated *in vacuo* . The residue was refluxed in 2% H_2SO_4 (5 mL) for 3 h. The cooled mixture was extracted with dichloromethane (2 x 30 mL), and the combined organic phases were dried and concentrated *in vacuo* to give a brown oil. Purification by flash chromatography[246] (85% hexane/15% ethyl acetate) gave 42 mg of **112** (7.5% yield) as a white crystalline solid whose physical constants were similar to those of **109**.

Degradation of ^{18}O-Labelled Terretonin to Methyl Benzoate (114) and GC/MS Analysis of 118

To a solution of 10.5 mg of terretonin (**104**) (22.4 µmol) in 0.5 mL THF at 0 °C was added 140 µL of n-butyllithium (1.6M solution in hexane, 224 µmol) with stirring. The solution was warmed to room temperature and stirred for 30 min. The resulting yellow suspension was cooled to 0 °C, 26 µL of benzoyl chloride (224 µmol) was added, and stirring was continued for 16 h. Benzyl alcohol (23 µL, 224 µmol) was added and the solution was stirred for an additional 3 h. The resulting suspension was partially purified by bulb to bulb distillation (50 °C, 0.1 mm Hg) to give 0.1 mL of distillate. This was diluted with 2.5 mL of ether and submitted for GC/MS studies. The GC/MS studies were carried out on a VG 7070E mass spectrometer coupled to a Varian Vista 6000 gas chromatograph fitted with a 30 m x 0.25 mm OV 351 capillary column. A flow rate of 1 mL/min of helium gas was used and a temperature gradient of 10 °C/min from 50 °C to 250 °C was applied. Two peaks were observed, that of benzyl alcohol (t_r = 8.37 min) and methyl benzoate (t_r = 9.97 min). Ratio of M^+ : $M^+ + 2$ for unlabelled methyl benzoate was

100 : 0.77, ratio of M^+ : $M^+ + 2$ for methyl benzoate derived from labelled terretonin was 100 : 1.86.

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